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Cardioprotective Pathways During Reperfusion: Focus on Redox Signaling and Other Modalities of Cell Signaling

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Abstract

Post-ischemic reperfusion may result in reactive oxygen species (ROS) generation, reduced availability of nitric oxide ($NO^{\bullet}$), Ca^{2+}overload, prolonged opening of mitochondrial permeability transition pore, and other processes contributing to cell death, myocardial infarction, stunning, and arrhythmias. With the discovery of the preconditioning and postconditioning phenomena, reperfusion injury has been appreciated as a reality from which protection is feasible, especially with postconditioning, which is under the control of physicians. Potentially cooperative protective signaling cascades are recruited by both pre- and postconditioning. In these pathways, phosphorylative/dephosphorylative processes are widely represented. However, cardioprotective modalities of signal transduction also include redox signaling by ROS, S-nitrosylation by $NO^{\bullet}$ and derivative, S-sulfhydration by hydrogen sulfide, and O-linked glycosylation with beta-N-acetylglucosamine. All these modalities can interact and regulate an entire pathway, thus influencing each other. For instance, enzymes can be phosphorylated and/or nitrosylated in specific and/or different site(s) with consequent increase or decrease of their specific activity. The cardioprotective signaling pathways are thought to converge on mitochondria, and various mitochondrial proteins have been identified as targets of these post-transitional modifications in both pre- and postconditioning. *Antioxid. Redox Signal.* 14, 833–850.

Introduction

ACUTE CORONARY ARTERY DISEASES are the leading cause of mortality and morbidity in Western countries. Cardiological practice has changed rapidly over the last few years, progressing from thrombolysis to direct coronary intervention and stenting to rapidly restore myocardial blood flow. It is generally accepted that whenever possible a short door-to-balloon time limits infarct size, ventricular dysfunction, and major cardiac adverse events (MACE), and, consequently, improves prognosis (68, 109, 120, 202). Therefore, it is no doubt that early reperfusion is the "gold standard" therapy for acute myocardial infarction. In fact, these strategies have had a major impact on improving outcomes. However, we have also come to understand that reperfusion itself is, paradoxically, an important cause of organ damage (68, 109, 202). Starting almost immediately after the restoration of blood flow, a cascade of adverse events (see below) triggers a vicious cycle leading to additional injury and cell death. Local and more widespread inflammatory responses will concur to increase the extent of infarction in otherwise viable tissue. This acceleration of damages after reperfusion led to the concept of *myocardial reperfusion injury* (3, 21, 68, 109, 156, 177, 202).

Myocardial Reperfusion Injury Is Due to Complex Mechanisms

Reperfusion injury is due to mechanisms involving mechanical, extracellular, and intracellular processes. The pathogenesis of reperfusion injury has been reviewed elsewhere (21, 53, 151, 156, 202, 207) and is beyond the aim of the present review. However, a short description of the principal events of the processes involved in reperfusion injury may be useful for the reader. Schematically, acute reperfusion injury can be due to the following interconnected key mechanisms: a) reactive oxygen and nitrogen species (ROS/RNS) generation; b) reduced availability of nitric oxide ($NO^{\bullet}$); c) Ca^{2+}overload; and d) mitochondrial permeability transition pore (mPTP) opening (Fig. 1).

These mechanisms may lead to cell death and to the activation of the NFκB and other transcription factors, which, in turn, lead to the augmented expression of molecules of cellular adhesion, leukocyte infiltration, and *no-reflow phenomenon*, which exacerbate tissue injury (see reviews 137, 177, 183, 201, 202, 207).

Reactive oxygen and nitrogen species generation

Reactive oxygen species (ROS) is a collective name for a group of oxygen-containing species such as superoxide anion

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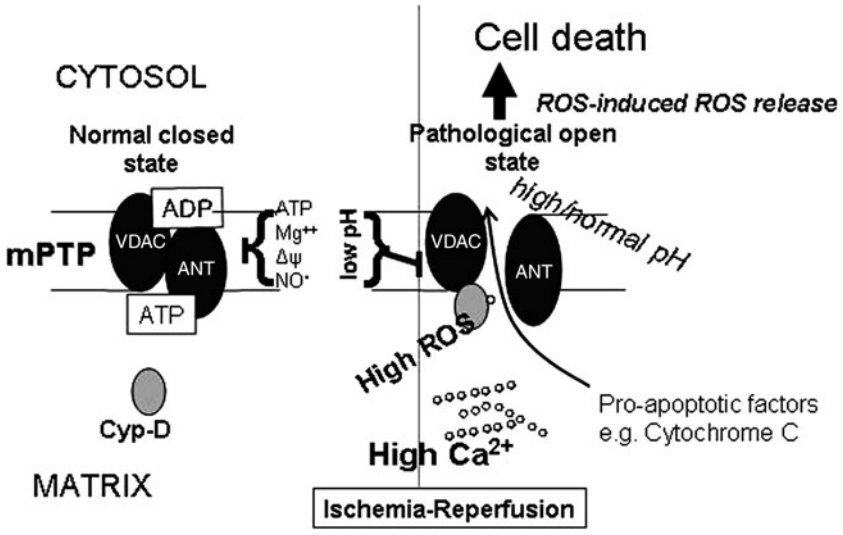

FIG. 1. Mitochondrial permeability transition pore (mPTP) is believed to be composed of the adenine nucleotide translocase (ANT) in the inner membrane, the voltage-dependent anion channel (VDAC) of the outer membrane, and cyclophilin D (Cyp-D) in the matrix. Ca^{2+} overload occurring during ischemia should bring mitochondria closer to the threshold at which mPTP opening takes place, favoring the occurrence of prolonged mPTP opening during reperfusion, a phenomenon described as mitochondrial priming. mPTP opening hardly occurs during ischemia, because it is strongly inhibited by acidosis. mPTP opening during reperfusion may be due to Ca^{2+} overload, which may stimulate the interaction of Cyp-D with other mPTP component(s), which triggers permeability transition. In fact, typically they open in reperfusion when Ca^{2+}overload, generation of reactive oxygen species (ROS), and pH normalization occur. Their opening leads to cell death through the release of pro-apoptotic factors and *via* ROS-induced ROS release. The *dashed vertical line* represents the passage from ischemia (low pH) to reperfusion (pH normalization).

radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$). Reactive nitrogen species (RNS) refers to the family of compounds derived from $NO^\bullet$, the signaling molecule synthesized from L-arginine by the nitric oxide synthases (NOSs, *i.e.*, neuronal, endothelial, and inducible NOS). $NO^\bullet$ can also be converted, by oxidation or reduction, into many chemically reactive forms including nitrogen dioxide radicals ($NO_2^\bullet$), peroxynitrite ($ONOO^-$), and nitroxyl (HNO), each of which yields distinct functional consequences (11, 12, 55, 138, 142, 164, 198). The implications of the biological balance between $NO^\bullet$ and oxidative stress have been examined in detail in several recent reviews (12, 55, 112, 138, 164, 198).

Various deleterious processes can be the result of an imbalance between the excess formation of ROS and/or RNS and limited antioxidant defenses (referred to as 'oxidative stress'). For instance, ROS indiscriminately react with lipids, proteins, and DNA. These are complex processes: in brief, ROS reaction with lipids gives rise to peroxyl and alkoxyl radicals leading to lipid peroxidation; reaction with amino acid residue side chains of proteins form protein carbonyls, and reaction with methionine forms methionine sulfide; reaction with DNA may oxidize DNA bases such as 8-OhdG, leading to mutation and DNA strand breaking (1, 12, 187, 204, 208–212). Excessive ROS/RNS production is considered deleterious to cell function and mitochondria, where they act as inducers of mPTP opening (see below). Alternatively, low levels of reactive species may act as secondary messengers, modulating signalling pathways by covalent modification of target molecules (referred to as 'redox signaling', see also below).

Oxidative stress related to the massive generation of ROS/RNS may play an important role in reperfusion injury (1, 12, 187, 204, 208–212). At reperfusion, $O_2^{-\bullet}$ and other ROS may strongly oxidize the cardiomyocytes already damaged by the ischemia, thus favoring cell death (1, 2, 53, 116, 183, 184). Moreover, in reperfusion, $O_2^{-\bullet}$ may react with $NO^\bullet$, forming $ONOO^-$. Therefore, $ONOO^-$, may represent a sign of a reduced availability of $NO^\bullet$ and it may participate with $O_2^{-\bullet}$ in myocardial injury (10-12, 52, 135, 165). In particular, high $ONOO^-$ concentrations are considered to be highly cytotoxic (11, 12, 135, 165). Peroxynitrite cytotoxicity is also due to its reaction with proteins (on tyrosine and thiol groups), lipids, and DNA (11, 12, 21, 135, 159). For instance, peroxynitrite inactivates prostacyclin synthase by tyrosine nitration, playing a crucial role in the pathophysiology of the cardiovascular system (211). The contribution of high levels of $ONOO^-$ to myocardial and vascular dysfunction during ischemia and reperfusion, and other cardiovascular pathologies has recently been reviewed (53, 135).

Although the sources of ROS and RNS within the cells are many, in cardiomyocytes the main sources are mitochondria (38–41,142). It has been suggested that for massive ROS release the involvement of mPTP is required. Zorov *et al.* (209) "triggered" massive ROS release in isolated cardiomyocytes *via* intracellular photoactivation of tetramethylrhodamine compounds. These triggered-ROS were associated with mitochondrial depolarization, along with prolonged mPTP opening and a consequent large burst of ROS from mitochondria. Thus, a positive feedback loop of "ROS-induced ROS release" has been suggested (209, 210). These organelles may also produce a large quantity of ROS from monoamine oxidase and p66Shc activity (39–41). Even NOSs can become "uncoupled", resulting in the generation of $O_2^{-\bullet}$ and $OH^\bullet$ instead of $NO^\bullet$ 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 (FAD and FMN) to O_2 is not inhibited, resulting, in fact, in a formation of $O_2^{-\bullet}$ and/or $OH^\bullet$ (212).

Cells may defend against excessive ROS by intracellular antioxidants, including $NO^\bullet$, and by the coordinated activity of scavenging enzymes. In particular, $O_2^{-\bullet}$ is transformed into H_2O_2 by superoxide dismutase (SOD) isoenzymes. However,

since in the presence of Fe^{2+} or Cu^{2+}, the H_2O_2 can be transformed into $OH^{\bullet}$, which may be more toxic than $O_2^{-\bullet}$ and H_2O_2, an increase in toxicity can occur. Yet, $O_2^{-\bullet}$ itself may trigger the release of Fe^{2+} from Fe^{3+}-complexes (Haber–Weiss cycle). Therefore, $O_2^{-\bullet}$ can be directly and selectively deleterious and/or it can be deleterious *via* its transformation into $OH^{\bullet}$. As the flux of $O_2^{-\bullet}$ exceeds the one of $NO^{\bullet}$, cellular damage may develop induced by one-electron oxidations caused by nitrogen dioxide and by the Fenton reaction (188). $O_2^{-\bullet}$ can also be transformed into $ONOO^-$, whose cytotoxicity can be reduced by the addition of $NO^{\bullet}$ *via* a secondary reaction due to the antioxidant properties of $NO^{\bullet}$ and formation of nitrosating species in this process (34, 52, 164). Yet the threat of $OH^{\bullet}$ can be avoided by the fact that H_2O_2 can be quickly reduced to water by several enzymes (catalase, glutathione peroxidase, and peroxiredoxins). It can, then, be argued that during reperfusion a limited formation of H_2O_2 may be protective if $NO^{\bullet}$ is adequately formed or added to reduce $ONOO^-$ cytotoxicity, *via* a secondary reaction, thus avoiding mPTP opening. This is in line with the observation that strategies to increase $NO^{\bullet}$ bioavailability (*e.g.,* by nitrite infusion (157)) in reperfusion are very often protective (*e.g.,* Refs. 169, 178–180) and is in line with the concept that when properly regulated ROS/RNS are not purely destructive (52), but rather engage an important cellular signaling function (see below, *Redox signaling by ROS and RNS*).

Reduced availability of nitric oxide

It is well known that $NO^{\bullet}$ can modify proteins via different chemical processes. Actually, $NO^{\bullet}$ exerts many of its physiological effects via the formation of complex (or coordinating) bonds with the transition metal ions of heme groups (94, 142, 198). This kind of reaction is the basis for the activation of classical vasodilatation, through a pathway wherein $NO^{\bullet}$ binds to soluble guanylate cyclase, thereby stimulating the formation of cyclic GMP (cGMP) from GTP. Subsequently, cGMP activates protein kinase G, which mediates many of the actions of $NO^{\bullet}$. It has been reported that physiological levels of $NO^{\bullet}$ inhibit mPTP opening with an IC_{50} of 11 n*M* (19). As said, when under pathological conditions $NO^{\bullet}$ production is not sufficient or it is supraphysiological this results in deleterious effects.

Clearly many of the pathological processes correlated to reduced availability of $NO^{\bullet}$ are due to the reduction of cGMP-dependent effects. However, nitration of tyrosine by RNS (not to be confused with nitrosylation, see below) may also play a role (34). In fact, as reported above, under pathological conditions $NO^{\bullet}$ reacting with $O_2^{-\bullet}$ can be just enough to form $ONOO^-$. Thus, peroxynitrite can react directly and irreversibly with proteins, lipids, and DNA, a result that is frequently deleterious (21). For instance, $ONOO^-$ irreversibly blocks the mitochondrial respiratory chain (108, 119). In fact, $ONOO^-$ reacts with sulfhydryl groups and iron–sulfur clusters in respiratory complexes, and induces nitration/oxidation of MnSOD, further augmenting mitochondrial oxidative stress in a vicious cycle (158).Yet, as said, when $NO^{\bullet}$ is sufficiently produced, $ONOO^-$ cytotoxicity can be reduced via a secondary reaction(164). $NO^{\bullet}$ deficiency can also cause vasoconstriction and formation of micro-thrombi into the lumen of the small vessels (1, 162, 169). These mechanisms, combined with the adhesion of the leucocytes to the endothelium, induced by ROS, can lead to the "no-reflow phenomenon" (1, 162). As a consequence, $NO^{\bullet}$ deficiency and free radicals also have 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. Upregulation of a program of pro-inflammatory genes will contribute to the onset and maintenance of post-ischemic inflammation (162, 163).

Ca^{2+} overload

In normoxia, Ca^{2+} acts at several levels within the mitochondria to stimulate ATP synthesis, and these organelles can be considered a major player in Ca^{2+} buffering. In fact, to generate a proton gradient across the inner membrane, Ca^{2+} uptake occurs in mitochondria that utilize oxygen (38–41). The cytosolic Ca^{2+} overload starts during ischemia and is further increased during reperfusion. In fact, the oxidative stress depresses Ca^{2+} regulatory mechanisms. Altered Ca^{2+} handling during ischemia induces structural fragility and excessive contractile activation upon reperfusion. The Ca^{2+} overload also activates calcium-dependent proteases, which could partially degrade contractile proteins. These effects are the basis of a progressive increase of ventricular diastolic pressure (hypercontracture), myocardial stunning, and contraction band necrosis (100, 151, 170). Ca^{2+} overload favors the expression of proapoptotic elements from mitochondria (208) and increases the cellular osmolarity, favoring explosive swelling of cardiomyocytes. Ca^{2+} overload may also occur within mitochondria where it is considered to be responsible for the prolonged opening of mPTP, which is considered the point of "no-return" leading to cell death (see below and Refs. 38–41, 66) Actually, mitochondrial Ca^{2+} overload can lead to enhanced generation of ROS, prolonged mPTP opening, rupture of the outer mitochondrial membranes (OMM) due to swelling, and cytochrome c release, thus leading to cell death (8, 32). The interplay between cytosolic Ca^{2+} and mitochondria is analyzed in more detail in other reviews of this Forum (14, 39, 84).

Mitochondrial permeability transition pore opening

The *mPTP* is a nonspecific megachannel of the mitochondrial membrane whose prolonged opening in the first few minutes of myocardial reperfusion promotes cell death (4, 8, 30, 32, 58, 60, 64, 69–72, 105). We should, however, keep in mind that mPTP opens and closes all the time and that a transient increase in opening probability of the pores may be involved in ROS-dependent cardioprotection by preconditioning (72, 105).

mPTP has been proposed to be formed by adenine nucleotide translocase (ANT), the voltage-dependent anion channel (VDAC), and cyclophilin-D (Fig.1) (38–41). Besides being the moment of "no-return" for cell death in reperfusion, mPTP opening is also involved in heart failure development (17, 58, 127, 196). The oxidative opening of mPTP is central in reperfusion injury (for reviews, see 38- 41,158), and, as said, it causes mitochondrial depolarization, loss of small molecular weight substances from the matrix, and rupture of OMM (6, 32, 126, 196). However, experiments with transgenic mice in each of the putative components of mPTP reached controversial results. In fact, neither gene deletion nor knockdown of

VDAC nor ANT prevents mPTP opening in response to mitochondrial Ca^{2+} overload (6, 9, 27, 97, 126). The intriguing possibility that cyclophilin-D has a role in regulating apoptotic proteins in a manner that is independent of the mPTP has been suggested (46). Nevertheless, in transgenic mice sustained modulation of a mitochondrial function may alter cardiac development and functions not related to the response to acute ischemia. Pharmacological tools to study the roles of mPTP in acute ischemia/reperfusion scenario are available, and could be applied clinically as therapeutic agents for ischemic heart disease (57–60, 86). In fact, selective targeting of drugs designed to modulate mitochondrial function and genomics for therapeutic benefit are made possible by recent advances in mitochondrial biology (for reviews, see Refs. 22, 86). More details on the central role of mPTP opening in protection and reperfusion injury are given by other reviews in this Forum (14, 29, 39).

In summary, conditions occurring during ischemia and reperfusion, such as Ca^{2+} overload, ROS, inorganic phosphate and mitochondrial depolarization favor prolonged mPTP opening (Fig.1). These factors are counteracted by physiological mPTP antagonists, such as increased mitochondrial membrane potential, low pH, magnesium ions, ADP, and nitric oxide (14, 39, 84).

In reperfusion, cell death can occur by apoptosis, oncosis/necrosis, and autophagy (see the following for extensive review on this topic (*e.g.*, Refs. 122, 134, 181)). However, autophagy is not simply a destructive phenomenon, but in certain conditions autophagy can be considered a protective mechanism against I/R injury (for reviews, see Refs. 62,155). Besides irreversible injury leading to cell death and myocardial infarction, reperfusion injury of the heart is characterized by reversible contractile dysfunction (stunning), endothelial dysfunction, microvascular damage, and reperfusion arrhythmias. All these deleterious events can be prevented by both pre- and postconditioning (190).

Preconditioning and Postconditioning

In 1986, Murry *et al.* (123) reported that four 5 min circumflex occlusions, each separated by 5 min of reperfusion, followed by a sustained 40 min occlusion (index-ischemia) dramatically attenuated I/R injury in the dog heart. This phenomenon was named ischemic preconditioning (PreC). In 2003, Zhao *et al.* (207) reported that three episodes of 30 sec of reperfusion/30 sec of ischemia performed immediately after index-ischemia (60 min coronary occlusion) in the dog heart dramatically attenuated reperfusion injury. This phenomenon was named postconditioning (PostC). The protective effects observed with PostC are comparable to those observed with the powerful PreC (109, 113, 207). In fact, PostC may reduce apoptosis, necrosis, and endothelial dysfunction/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 (56, 96, 125). Notable, already by 1994 Grech and Ramsdale (63) had reported that intermittent reperfusion may restore sinus rhythm. Whether PostC protects from myocardial stunning is still controversial (148, 172). These protective effects are discussed in the review of Vinten–Johansen *et al.* (190).

PostC, first described in dogs, was subsequently demonstrated in other species, and can also be recruited clinically in human patients to reduce infarct size (59, 73, 86, 140, 176). Notably in humans, PostC has long-term protective effects (86). The infarct size reduction by ischemic PostC depends on duration of the index-ischemia, on the number and duration of the brief re-occlusions, and/or on the presence of comorbidities (81, 148, 174). Further studies should resolve the difference in efficacy of PostC between experimental models and human subjects with comorbidities and/or aging (40, 149, 155, 190).

Besides passive effects attributed to modification of hemodynamics during PostC maneuvers, the cardioprotection triggered by PostC is attributed to active effects that are mediated by several signaling pathways (77, 139–141, 144, 147, 191). The link between passive and active effects is not clear, but during initial reperfusion PostC delays, the post-ischemic recovery of intracellular pH, and the ability to trigger the activation of biochemical pathways and to limit infarct size might depend on this pH effect [see below and Inserte *et al.* (84)]. Potentially, in these activated pathways, variations of protein structures/activities occur via different post-translational modifications, namely phosphorylation, dephosphorylation, O-linked glycosylation, S-nitrosylation, and sulfhydration (see below, *Modalities of cell signaling in cardioprotection*). In this review we will focus on these mechanisms of cardioprotection with emphasis to redox-signaling to reduce reperfusion injury.

Before analyzing these mechanisms, it is necessary to review a brief description of the main cardioprotective pathways involved in pre- and postconditioning, which have been described in several recent publications (59, 68, 71, 140, 146, 201). Also in this Forum up-to-date, aspects of the pathways involved in PostC are reported (*e.g.*, 29, 70).

Cardioprotective Pathways

It is now thought that, after a triggering phase in preischemic period, the actual protection by PreC occurs in the reperfusion rather than ischemic phase with the repopulation of sensitized G-protein coupled receptor (GPCR) at the beginning of myocardial reperfusion following the index-ischemia. Thus, pre and postconditioning activate cardioprotective pathways that are protective against reperfusion injury. A schematic representation of these cardioprotective signal transduction pathways is reported in Figure 2.

Both PreC and PostC engage GPCR and enzymes that converge on the mPTP. However, subtle differences are present in the pathways and in cell signaling at different times and different species (14–16, 29, 70, 77, 173). GPCR agonists include adenosine, bradykinin, and opioids, with a possible sensitization of relevant receptors by prior ischemias. The exact subtypes of adenosine, opioids, and bradykinin receptors involved in triggering protection are still a matter of controversy (29, 142). We have shown a role for endogenous bradykinin in PostC via B_2-bradykinin receptor activation (141, 144, 200). A role for these receptors in PostC has been confirmed in other models, including a knockout mice model (142, 200). Receptor tyrosine kinases may also be involved, possibly via the epidermal growth factor receptor transactivation to activate PI3K/Akt. The activation of PKC can be downstream of Akt or, especially in the triggering phase, can be achieved *via* phospholipase signaling with adenosine receptor agonism (65, 73, 134, 201). For the aims of the current

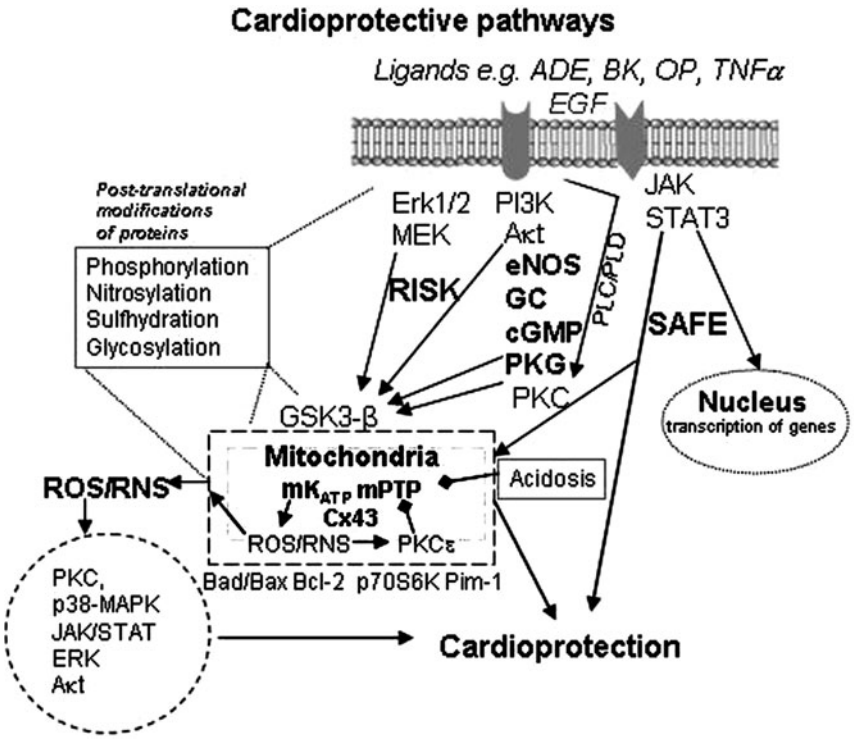

FIG. 2. Schematic representation of cardioprotective pathways (Reperfusion Injury Salvage Kinases (RISK) pathway and Survivor Activating Factor Enhancement (SAFE) pathway). Post-translational modifications of proteins occur both in the cytosol and in organelles, particularly within mitochondria in which specific targets are recognized. ADE (adenosine), OP (opioids), TNFα (tumor necrosis factor alpha), PLC (phospholipase C), and PLD (phospholipase D), BK (bradykinin), EGF (epidermal growth factor); other acronyms as in the text. Persistent acidosis during early reperfusion, activation of RISK and SAFE pathways, and the activation of other cell signaling lead to cardioprotection, where the prevention of mPTP opening plays a central role. Reactive oxygen and nitrogen species (ROS/RNS) with signaling role may affect other cell signaling. Although sulfhydration and glycosylation may occur in reperfusion, they are not reported in ischemic postconditioning. STAT3 is a transcription factor and thus nuclear effects are expected. Yet, it has also direct effects on mitochondria (see review by Hausenloy *et al*. (70) in this Forum). For further explanation, see text.

review, the involvement of tyrosine kinases is particularly intriguing since these kinases are activated by low doses of hydrogen peroxide limiting reperfusion injury (166).

Collectively three major pathways are recognized: the cGMP/PKG-pathway, the Reperfusion Injury Salvage Kinases (RISK) pathway and Survivor Activating Factor Enhancement (SAFE) pathway (65–73, 102, 139–144, 147, 191, 206) (Fig.2). RISK includes the phosphorylation of Akt, ERK1/2, p70S6K, and NOS. SAFE pathway includes JAK and STAT-3 phosphorylation, and both pathways may converge on mitochondria *via* GSK-3β, which is suggested to be among the end-effectors for the cardioprotection by ischemic PostC (70–72,185). Other proteins regulating apoptosis, such as Bad/Bax, Bcl-2, and Pim-1, are also correlated with cardioprotection *via* prevention of mPTP opening and reduction of mitochondrial swelling. Overall, various protective mechanisms seem to converge on mPTP (72, 142, 146). Contextually other enzymatic (phosphorylation and O-linked glycosylation) and nonenzymatic (nitrosylation and sulfhydration) intracellular signaling may involve multiple targets, which converge on organelles, particularly mitochondria, which are considered critical events leading to protection (26, 70, 139–144, 167, 186). These pathways and intracellular signaling are not mutually exclusive and are recruited by both pre- and postconditioning to a different extent, depending on a plethora of factors. In this perspective it is particularly intriguing that VDAC, constituent of the mPTP, may be also a target for O-linked glycosylation (see below). Therefore, apart from cGMP/PKG, RISK, and SAFE pathways, there are redox mechanisms and events that may be considered alternative or cooperative to the classical pathways. Of note, the importance of RISK has been recently questioned, and its relevance in larger mammals seems to be severely limited; from a translational point of view these animal models may be more suitable (76, 131, 173). Concerns and controversies about the possibilities of translating the results of animal experiments into human clinical trials have recently been discussed by van der Worp *et al.* (189). Nevertheless, preliminary clinical data indicate that drugs targeting RISK may confer a benefit to patients with acute myocardial infarction over and above that provided by myocardial reperfusion alone (70), but they remain to be confirmed in large-scale clinical studies. These features are also considered by Ivanes *et al.* (86) and by Vinten–Johansen *et al.* (190) in the present Forum.

Interaction Between Redox Environment and Cardioprotective Pathways

ROS/RNS with a signaling role are suggested to be formed during three time points: during preconditioning-ischemia and/or during reperfusion that follows the brief preconditioning-ischemia, as well as in pre and postconditioning during the initial part of reperfusion that follows the index-ischemia (Fig. 3) (30, 73, 142).

The origin of these protective reactive species can be many, including mitochondria, in which K^+ATP (mK_{ATP}) channels, which are considered targets of protective cascades (14, 142, 161, 168, 201), may play a pivotal role. In fact, it has been suggested that mK_{ATP} channel activation leads to alkalinization of the mitochondrial matrix and generation of ROS/RNS with a protective signaling role (33, 142, 168, 201). It has also been suggested that NO donors activate mK_{ATP} channels in rabbit ventricular myocytes and potentiate the protective effect of mK_{ATP} opener diazoxide (168). However, controversy exists on the nature, existence, and opening of mK_{ATP} channels, which may also be a toxic process (33, 36). Nevertheless,

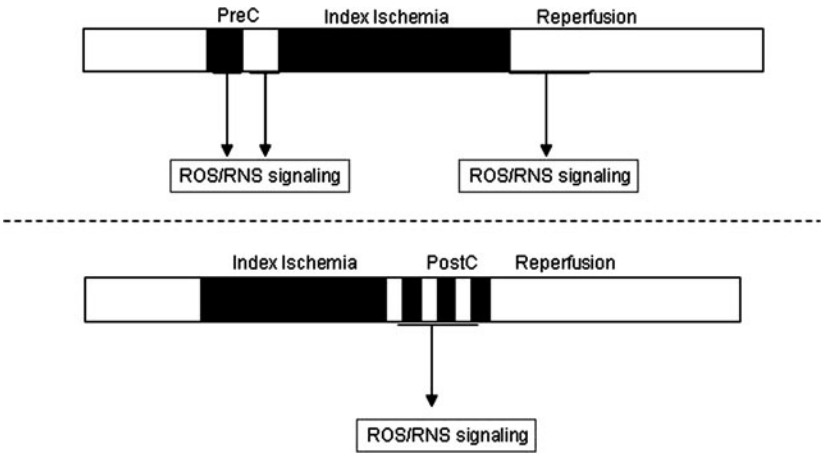

FIG. 3. Reactive oxygen and nitrogen species (ROS/RNS) with signaling role have been described during brief preconditioning ischemia, during reperfusion following the brief preconditioning ischemia, as well as during early reperfusion which follows the index-ischemia, both in preconditioning (PreC) and postconditioning (PostC).

it has been suggested that besides channel phosphorylation, mK_{ATP} can be opened by $NO^{\bullet}$ and derivative (nitrosylation) as well as by sulfhydration (see below). Moreover, mitochondrial connexin-43 has also been implicated in ROS signaling although its role is not completely defined (14–16). A target of ROS in 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 (184). Indeed, 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 (98). RNS-dependent activation of PKC, possibly via redox-sensible nitrosylation process, has been also suggested (161, 180). Actually, PreC via ROS/RNS formation leads to a decreased intracellular reduced glutathione (GSH) pool, which may stabilize S-nitrosothiol formation by attenuating GSH-mediated trans-/denitrosylation, thus potentiating redox signaling and converting death-signal into survival-signal (161, 167).

Although PKC plays a pivotal role in cardioprotection, PKC location in the signaling cascade is unclear (69, 70). Yet the regulation and role of PKC is still debated, with a possible role for directly activated PKC, together with possible PKG-dependent phosphorylation of mitochondrial PKCε, which in turn inhibits mPTP opening *via* ROS signaling (57, 201).

Generation of ROS/RNS, distal to mK_{ATP} opening, may also trigger activation of kinases additional to PKC, including p38-MAPK and JAK/STAT (15, 70, 73, 201). The involvement of p38-MAPK and JNK in preconditioning was also reported for angiotensin-II and summarized in the recent review by Daiber (33). Besides kinase activation, the increased phosphorylation may also be the result of the well-known inhibitor effect of ROS on phosphatases (for review, see Ref. 24).

We and other authors have shown that ROS scavengers such as N-acetyl-cysteine (NAC) and N-(2-mercaptopropionyl)-glycine (MPG) given during the PostC maneuvers prevented the protective effects (43, 142, 150, 186). These scavengers given in the first few minutes of myocardial reperfusion following the index-ischemia also prevent PreC-protection (73). Variations of the activity of endogenous antioxidant enzymes in early reperfusion may also play an important role in PostC protection (150). Another mechanism in both pre- and postconditioning involves the persistence of intracellular acidosis during early reperfusion (30, 54, 73, 83, 86). In fact, while acidosis prevents mPTP-opening, the reintroduction of O_2 at the beginning of reperfusion may allow ROS signaling to activate protective cascades. These may also prevent the reactivation of mitochondrial enzymes able to produce massive quantity of ROS. Of note, acidosis is also responsible of nonenzymatic $NO^{\bullet}$ formation (213), which has been suggested to be involved in PostC-triggering (142).

Ultimately, the mPTP-opening during early reperfusion is inhibited by many of the above reported effects. In particular, persistent acidosis during early reperfusion may play a pivotal role, contributing to the limitation of mPTP-opening and additionally favoring enzymatic and/or nonenzymatic mechanisms of protection. Therefore, it can be argued that phosphorylative processes and/or other modalities of signal transduction (*e.g.,* S-nitrosylation, S-sulfhydration, and O-linked glycosylation) are recruited by both pre- and postconditioning to a different extent to achieve protection *via* mitochondrial salvage.

Importantly, the cardioprotection by pre- and postconditioning can be mimicked pharmacologically by the administration of endogenous ligands, such as adenosine, bradykinin, or opioids, and by agents that act on mitochondria such as diazoxide (141, 142, 144). Particularly intriguing is the protective role of cyclosporine A, an inhibitor of mPTP-opening, which has recently been used in humans during reperfusion as a PostC agent (59, 86). It is also of note that redox-sensitive cardioprotective effects can be induced by nitroglycerin (31, 44) and by nitrite infusion (157).

Modalities of Cell Signaling in Cardioprotection

Cellular signaling or signal transduction pathways represent a common property of viable cells, which include signals starting from enzymes, cyclic nucleotides, calcium, and even gases such as carbon monoxide, hydrogen sulfide (H_2S), and $NO^{\bullet}$. Although protein phosphorylation is the most widely studied form of signal transduction, as above mentioned, there are many other post-translational modifications of proteins (e.g., acetylation, glycosylation, glutathionylation, methylation, nitration, nitrosylation, and ubiquitylation) (35, 79, 88, 92). As said, some of these "additional" modalities of signal transduction have been described in the ischemia/reperfusion scenario and have already shown their importance in cardioprotection. Of course, these include redox

signaling in which ROS/RNS modify proteins in a meaningful way, including *S*-nitrosylation by $NO^{\bullet}$ and derivative. *S*-sulfhydration by H_2S is also gaining ground as a cellular signaling process in cardioprotection. Other interesting signaling systems that are still poorly understood are O-linked glycosylation, which involves the linkage of beta-N-acetylglucosamine through an oxygen atom to proteins at their serine and threonine residues (23, 42, 105, 106, 130). Theoretically, all these modalities are not limited to interactions with specific enzymes but rather act to regulate an entire pathway influencing each other. For instance, an enzyme can be phosphorylated and/or nitrosylated in a specific and/or different site(s) with consequent increase or decrease of its specific activity. Despite this interplay, for clearness sake, in the following part we will consider singularly the most relevant cellular signaling in cardioprotection (*i.e.*, those *enzymatic-mediated:* phosphorylation and O-linked glycosylation, and those *nonenzymatic-mediated:* redox signaling, including S-nitrosylation and S-sulfhydration). Whenever possible, interactions among these signaling processes are outlined. Of course, each of these processes has been extensively reviewed elsewhere (21, 35, 79, 80, 87, 88, 90, 98, 180, 193); therefore here they are briefly analyzed with particular attention to the cardioprotective mechanisms.

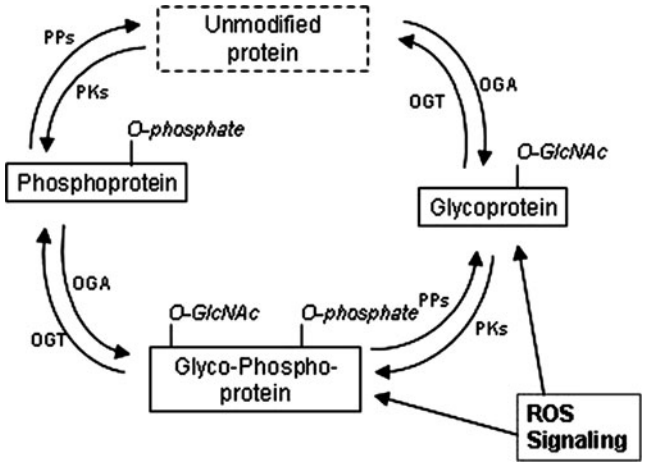

FIG. 4. Proteins can be O-linked glycosylated (O-linked glycosylation, O-GlcNAc) and/or phosphorylated, and the adjacent O-GlcNAc and/or phosphorylation can influence the addition of either moiety. An increase in the production of mitochondrial reactive oxygen species (ROS) with signaling role may be accompanied by an increase in O-GlcNAc. Uridine-diphospho-N-acetylglucosamine: polypeptide β-N-acetylglucosaminyltransferase (OGT); N-acetylglucosaminidase (OGA); Protein kinases (PKs); Protein phosphatases (PPs). See text for further explanation.

Enzyme-Mediated Post-Translational Modifications in Cardioprotection

Phosphorylation

The processes of phosphorylation of proteins as regulatory modalities in the cell have been known since the early 1950s (20). In ischemia/reperfusion and cardioprotection, a plethora of kinases and phosphatases are involved (see above and Refs. 14, 29, 70, 190). Processes of phosphorylation/dephosphorylation occur on the cell membrane, in the cytosol, and in organelles (Fig. 4). In particular in the mitochondria, which are considered the final target of cardioprotective pathways (14), phosphorylation/dephosphorylation occurs on the external face of the OMM, in the intermembrane mitochondrial space (IMS), and in the mitochondrial inner membrane (MIM) and matrix.

As said, RISK and SAFE pathways consider phosphorylative cascades outside mitochondria (70, 71, 102, 185). Sphingosine-1-phosphate (S1P), which plays a pivotal role in cytoskeletal rearrangement and apoptosis, also seems necessary for successful preconditioning. Recently it has been shown that sphingosine kinase-1, the key enzyme catalyzing the formation of S1P, is critical for cardioprotection by PostC (89). Apart from the phosphorylative events in the cell membrane and cytosol, converging lines of evidence support that pathways converge on mitochondria, where phosphorylation has an impressive impact on cardioprotection. Therefore, here we focus on phosphorylation within mitochondria in the cardioprotective scenario [The role of mitochondrial function in Pre and PostC is reviewed in other articles of the present Forum (14, 39)].

On the external face of the OMM, there are several intracellular receptors (scaffolds), which serve as sites for the integration of signals for different processes (*i.e.*, apoptosis, cytoprotection, energetic demand, fission, and steroidogenesis) (175). Among scaffolds, we consider the PKA anchoring proteins (199), and the PKC-interacting proteins (152) RICKs and RACKs (*i.e.*, the receptors for inactive/active kinases, respectively) (118). For instance, both PKCδ and PKCε mitochondrial translocation have been seen to be involved in ischemia/reperfusion injury and cardioprotection, respectively (28). Studies with cardiomyocytes have shown that inhibitors of the PKC–RACK interaction abolish cardioprotection against ischemia/reperfusion (25) as well as the associated phosphorylation of mitochondrial targets (132). PKCε translocates to the mitochondria where it phosphorylates VDAC with consequent inhibition of the mPTP (7, 73). Yet, mitochondrial PKCε was found in complexes with MAP kinases. Formation of these complexes correlates with phosphorylation/inactivation of the pro-apoptotic protein BAD (8, 205). PKA can also link to the OMM through one of the PKA anchoring protein (D-AKAP1) (67). Aside from several isoforms of PKC and PKA, other kinases that have been shown to undergo translocation to the OMM include GSK-3β, Raf kinases, and ERKs. In particular, ERK1/2 may associate with the OMM in a complex with PKCε, though their substrates have not been defined (8). It has also been suggested that signalosomes (scaffolded caveolins containing enzymes) triggered by cardioprotective ligands converge on OMM to activate mK_{ATP} and to prevent mPTP-opening (57).

In the IMS, principal targets for phosphorylation are mainly involved in bioenergetics and apoptosis. These processes are particularly relevant in cardioprotective mechanisms. The majority of phosphorylation in the IMS appears to be on tyrosine residues. Given their location, phosphorylation of these tyrosines might be predicted to influence nucleotide transport (104). For instance, phosphorylation of ANT (component of mPTP) at its Tyr194 has been found in rat hearts either pre- or postconditioned with isofluorane (51). Aside the IMS-facing cavity of ANT, other important proteins of the IMS have emerged as phosphoproteins, including cytochrome c,

creatine kinase, and Ser 115/116 of subunit I, as well as sites within matrix-facing subunits IV and Vb (50). In studies of myocardial I/R injury, hypoxia reduced complex IV function coincidentally with hyperphosphorylation (Ser, Thr, and Tyr residues) of several subunits on complex IV, including subunit 1. Complex IV activity could be rescued by PKA inhibitors (74, 155).

In the MIM and matrix, proteins fall into several functional categories, including energy metabolism/oxidative-phosphorylation, lipid metabolism, and redox enzymes; frequent targets are pyruvate dehydrogenase complex, branched chain dehydrogenase, F_1F_0ATPase, and complex I. In the context of cardioprotection, pharmacological pre- and postconditioning with either adenosine or diazoxide led to phosphorylation of many mitochondrial proteins. Studies suggested at least five phosphorylation sites on the β subunit of the F_1F_0ATPase (94). Inhibition of F_1F_0ATPase activity may be a feature of the preconditioned heart, thus reducing ATP consumption by reverse mode of the enzyme (145). In the MIM of subsarcolemmal mitochondria, the phosphorylated portion of connexin-43, which plays an important role in ROS formation, increases with ischemia and decreases with PostC (16, 146). From proteomic studies it appears that there are more mitochondrial phosphoproteins than previously thought, particularly within the matrix and on the matrix-facing proteins of the inner mitochondrial membrane (78, 136), further supporting that phosphorylative processes occur within the mitochondria (57,142). Therefore, our better understanding of mitochondrial phosphorylation will also be of benefit for a future better understanding of the cardioprotective phenomena.

O-linked glycosylation (O-GlcNAcylation)

There are two basic types of glycosylation that occur on either asparagines (N-linked) or serines and threonines (O-linked) residue of proteins. Linkage of beta-*N*-acetylglucosamine through an oxygen atom (O-linked) to proteins appears to be the most relevant in cardioprotection. While phosphorylation is determined by a plethora of enzymes, O-linked glycosylation is controlled by two enzymes, a *transferase* that attaches the sugar group (uridine-diphospho-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyltransferase, *OGT*) and a *hexosaminidase* that removes it (beta-N-acetylglucosaminidase, *OGA*) (Fig. 4). It has been observed that ROS may increase flux through the hexosamine biosynthetic pathway, resulting in greater O-GlcNacylation of proteins (101, 160).

On several proteins, O-GlcNAcylation and O-phosphate alternatively occupy the same or adjacent sites (the "yin–yang phenomenon"), leading to the hypothesis that one function of this sugar group is to transiently block phosphorylation (197). Due to the importance of phosphorylation in cardiac protection, one can speculate that O-GlcNAcylation may interfere with protection. However, Champattanachai *et al.* (23) demonstrated in isolated neonatal rat ventricular myocytes that an inhibitor of OGT markedly reduced O-GlcNAcylation levels and exacerbated I/R injury. The authors suggested that increased O-GlcNAcylation levels may attenuate I/R induced Ca^{2+} overload and that activation of metabolic pathways leading to an increase in O-GlcNAcylation levels is an endogenous stress-activated response that improves cell survival. It has been shown that glutamine-induced protection of isolated rat heart from I/R injury is mediated via increased protein O-GlcNAcylation levels (107). In particular, 30-min pretreatment with glutamine significantly improved cardiac functional recovery and decreased cardiac troponin-I release during reperfusion. This cardioprotection has been attributed to elevated levels of O-GlcNAcylation on nucleo-cytoplasmic proteins. Ngoh *et al.* (129) have shown in isolated cardiomyocytes subjected to hypoxia/reoxygenation that elevated expression of OGA reduced O-GlcNAcylation levels and augmented post-hypoxic cell death. Yet, short interfering RNA directed against OGA, as well as pharmacological inhibition of this hexosaminidase, significantly augmented O-GlcNAcylation levels and reduced hypoxia/reoxygenation cell death. Intriguingly, Ngoh *et al.* (129, 130) showed that the mitochondrial VDAC is also a target for O-GlcNAcylation modification and that the inhibition of OGA activity improves, whereas augmentation impairs, mitochondrial membrane potential recovery.

It should be emphasized that the interplay between these two post-translational modifications of proteins is not always reciprocal; rather a cross-talk between phosphorylation and O-GlcNAcylation signaling exists. For example, some proteins can be concomitantly O-GlcNAcylated and phosphorylated, and the adjacent O-GlcNAc or phosphorylation can regulate the addition of either moiety (Fig. 4). Other proteins, such as GSK-3β, have multiple and distinct phosphorylation/O-GlcNAcylation sites on the same protein. Interestingly, phosphorylation/inhibition of GSK-3β, which is protective, increased O-GlcNAcylation of 10 proteins, but decreased O-GlcNAcylation of 19 proteins (195). Conversely, increasing global O-GlcNAcylation levels by OGA inhibition resulted in a decrease in phosphorylation at about 33% of sites and an increase in phosphorylation at about 18% of sites (194). These studies clearly demonstrate nonreciprocal interplay between O-GlcNAcylation and phosphorylation and indicate the possibility for cooperativity between these types of cell signaling.

A cross-talk between redox and O-GlcNAcylation signaling has also been described (101). This may have a strong relevance in cardioprotection; in fact an increase in mitochondrial ROS production may be accompanied by an increase in O-GlcNAcylation (Fig. 4) (45), which, in turn, leads to an increase in O-GlcNAcylation of mitochondrial proteins and increased tolerance of mitochondria to stress. Some evidence indicates a potential mitochondrial isoform of OGT (93, 128, 130, 131). Moreover, it has been reported that augmentation of O-GlcNAcylation levels in cardiomyocytes attenuates H_2O_2-induced loss of mitochondrial membrane potential (23, 93, 128, 131) and that this may be due to O-GlcNAcylation of mitochondrial proteins such as VDAC. All these studies suggest that ROS from mitochondria contribute to the regulation of O-GlcNAcylation of proteins, which could, in turn, modulate the mitochondria response to oxidative-stress. Of course to establish the real role of O-GlcNAcylation in the contest of cardioprotection will require much more research. For instance, though O-GlcNAcylation variations have been observed in reperfusion, to the best of our knowledge, nobody has studied the role of O-linked glycosylation in PostC. Finally, it must be pointed out that although acute O-GlcNAcylation is beneficial, chronic O-GlcNAcylation may promote myocardial apoptosis (160).

Nonenzymatic-Mediated Post-Translational Modifications in Cardioprotection

Redox signaling by ROS and RNS

When properly regulated under physiological conditions, both ROS and RNS are not purely destructive, but rather engage in important signaling functions both in and outside of mitochondria. Collectively with redox signaling, we consider the fact that proteins may undergo reversible chemical changes in response to changes in local redox potential. The ROS-induced cellular responses include precise molecular signals (modifications of cellular redox state) and their transduction into the organelles and the nucleus (compartmentalization), which modifies their (patho)-physiological role with a consequent influence on functions such as resistance to stress, senescence, and programmed cell death.

Here we focus on the oxidative processes (oxidation) of proteins, and in particular, on the modification of the cysteine thiol by incorporation of a $NO^{\bullet}$ moiety to a sulfur atom to form the S–NO bond (S-nitrosylation). Since it has been suggested that nitric oxide is a weak S-nitrosating agent, it is likely that to yield S-nitrosothiols an incorporation of NO^{+} (nitrosyl cation) occurs. At high concentrations in oxygenated solutions, $NO^{\bullet}$ may mediate S-nitrosylation *via* N_2O_3 formation by the autoxidation of $NO^{\bullet}$ to nitrogen dioxide radicals and subsequent reaction with another $NO^{\bullet}$. The instantaneous redox state and ultrastructural accessibility of cysteine residue(s) under low-oxygen tension, such as hypoxia and ischemia, might determine whether a particular thiol in a given protein is subjected to S-nitrosylation (167).

As said, low amounts of ROS generated during brief periods of ischemia/reperfusion or given in lieu of brief periods of ischemia/reperfusion have been reported to be responsible for preconditioning-triggering (48, 183, 184). The necessity of ROS signaling was demonstrated by both inducing PreC with ROS generators and avoiding PreC with ROS-scavengers given before the index-ischemia. Also, many G-protein-coupled receptor activators trigger preconditioning-like protection *via* ROS-dependent mechanisms, which may include PKC activation (71, 73, 121, 122, 142, 198, 201, 202).

We were the first to consider that ROS could also be included among the triggers of PostC. In fact, as said, large spectrum ROS scavengers such as NAC and/or MPG given during the PostC maneuvers prevented the protective effects (43, 142, 147, 186). Yet, we have been unable to reproduce cardioprotection with ROS generation by purine/xanthine oxidase given at reperfusion (141). Since ROS scavengers, given at the beginning of reperfusion, abolished both pre- and postconditioning-induced protection (72, 141, 153, 186), it is likely that the type, the concentration, and/or the compartmentalization of reactive species may play a pivotal role in triggering protection at reperfusion time. We can not exclude that a different ROS generator could trigger PostC protection (142). Postconditioning increased cardiac 3-nitrotyrosine concentration (a marker for $ONOO^{-}$ formation) after 5 min of reperfusion in normal but not in cholesterol-fed rats. Thus, early increase in $ONOO^{-}$ after PostC may play a role in PostC-protection (99). However, hyperlipidemia enhances preischemic levels of $ONOO^{-}$(133), but alters the PostC-induced $ONOO^{-}$ formation and blocks the protective effect (99). Furthermore, Iliodromitis *et al.* (81) and Wang *et al.* (192) suggest that PostC may reduce $ONOO^{-}$ formation. In particular, Iliodromitis *et al.* (81) report that PostC reduces myocardial and circulating levels of 3-nitrotyrosine after 10 min of reperfusion in the normal, but not in the hyperlipidemic rabbits. We have shown in rat hearts that, after 7 min of reperfusion, PostC induces an increase in S-nitrosylation of proteins and a reduction in 3-nitrotyrosine levels (150). To reconcile these apparent opposing results (81, 99, 150, 192) we can speculate that after an initial increase in $ONOO^{-}$, a further increase in $NO^{\bullet}$ (via enzymatic and nonenzymatic processes (213)) can lead to the formation of N_2O_3, thus lowering $ONOO^{-}$ level and increasing S-nitrosylation (see also below). In fact, a decrease in $ONOO^{-}$ levels in the presence of excess $NO^{\bullet}$ may be due to the secondary chemical interactions occurring between $NO^{\bullet}$ and $ONOO^{-}$ (164). Many different endogenous enzymes regulate the intra-tissue homeostasis of ROS, including SOD and catalase (142, and references therein). We found that PostC discretely changes the activity of these enzymes in early reperfusion, decreasing SOD and increasing catalase activity (150). These effects might shift reactions towards RNS formation and may have impact on S-nitrosylation thus reducing injury due to oxidative-stress (see below and Figs. 5 and 6).

In this context, the sulfhydryl chemistry of cysteine residues within proteins represent peculiar targets, as they can adopt multiple oxidation states (75). In fact, cysteines can react with ROS/RNS to yield a number of species including intra- and intermolecular disulfide bonds (disulfide cross-linking), S-nitrosylated proteins, or the formation of mixed disulfides with glutathione (glutathionylation) (Fig.5) (79, 87). For instance, oxidation/nitrosylation/glutathionylation also represents intra-mitochondrial redox signals. In fact, experiments have shown that low concentrations of H_2O_2 and S-nitroso-N-acetylpenicillamine cause redox modification of a selected group of mitochondrial proteins, despite little change

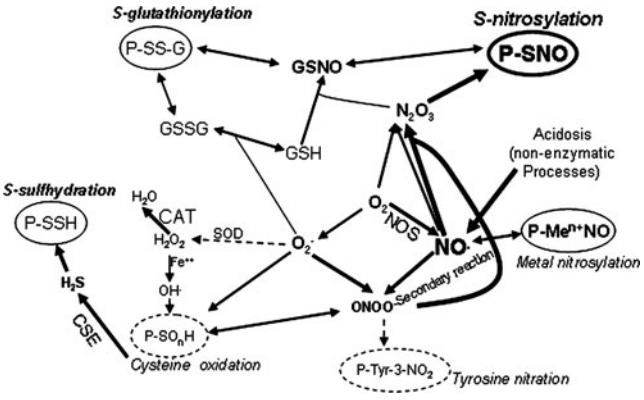

FIG. 5. Chemical relationship among different reactive oxygen species (ROS), reactive nitrogen species (RNS), and hydrogen sulfide (H_2S), and their impact on some post-translational modification of proteins. The processes, the enzyme activities, and the reactions increased by post-conditioning (PostC) are in *thick bold lines*, those decreased by PostC are in *dashed/thin lines*. In the excess of $NO^{\bullet}$, the secondary reaction between $ONOO^{-}$ and $NO^{\bullet}$ may favor S-nitrosylation. H_2S physiologically produced by CSE from cysteine, induces S-sulfhydration of proteins. GSH, glutathione; GSSG, glutathione disulfide; GSNO, S-nitrosoglutathione. See text for further explanation and other acronyms.

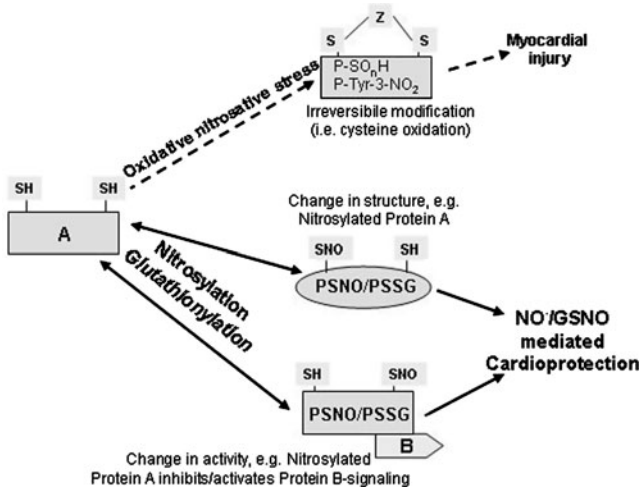

FIG. 6. Nitrosylation and glutathionylation are *reversible* post-translational protein modification leading to protection; oxidative/nitrosative stress may lead to *irreversible* modifications of protein which leads to damages. See also Figure 5.

in the redox status of the global mitochondrial thiol pool (79). Several of these proteins were recognized as redox-modified in response to endogenous mitochondrial ROS formation. Proteins targeted included pyruvate-dehydrogenase-kinase-2 and propionyl-CoA-carboxylase, among others. Both enzymes were inhibited by oxidation and could be reactivated by reduction with dithiothreitol (DTT). It can be inferred that, though many mitochondrial proteins can be thiol-modified under oxidative-stress, only a small subset of these modifications can be considered a real physiological/reversible redox signal and that the primary distinction between them would appear to be site-specific. In this context, S-nitrosylation and glutathionylation of proteins can be considered pivotal signaling in cardioprotection. Due to space constraint, we focus on S-nitrosylation. Useful information on the molecular mechanisms and potential clinical significance of S-glutathionylation can be found in the recent review by Dalle–Donne *et al.* (35).

S-Nitrosylation

Nitric oxide can be generated by enzymatic and nonenzymatic processes (213). Incorporation of $NO^{\bullet}$ moieties by covalent bonding to protein groups is chemically possible in the case of cysteine thiols, tryptophan indols, and amines (lysine and N-terminal) (172, 204), although S-nitrosylation (*i.e.*, the S–NO bond) can be considered the most important in the ischemia/reperfusion scenario, because of its higher reactivity, its occurrence in biological systems, and its influence on many protein functions. In fact, S-nitrosylation has emerged as an intriguing signaling modality, independently of the induction of cGMP production by $NO^{\bullet}$, effectively acting as a *reversible* molecular switch analogous to phosphorylation. From a terminology viewpoint, the incorporation of $NO^{\bullet}/NO^{+}$ moiety to a thiol can be clearly individuated because of the prefix "S-", referring to the incorporation of the moiety to a sulfur atom to form the S–NO bond. The term *nitration*, instead, describes the incorporation of nitrotriatomic group ($-NO_2$) at position 3 of the phenolic ring of tyrosine residues (Fig. 5). Although nitration may be a step of protective pathways (99), this modification is clearly related to the formation of peroxynitrite in cardiovascular diseases (for reviews, see Refs. 85, 139, 187).

Due to the complexity of $NO^{\bullet}$ and thiol chemistry, there are many possible reaction mechanisms that can lead to S-nitrosylation (Fig. 5), although which one prevails *in vivo* is still debated (79, 204). Of course, a complete view of the role of $NO^{\bullet}$ in cardioprotection must now include these cGMP-independent/redox-dependent along with "classical" cGMP-dependent mechanisms. Indeed, the role of $NO^{\bullet}$ in the cardioprotection afforded by PreC (21, 92) and PostC (139, 142) has been well documented. Until recently, however, many studies have centered on elucidating the role of classical $NO^{\bullet}$ signaling through cGMP to the mitochondria (140). Recently Sun *et al.* (178–180) have shown that PreC may cause S-nitrosylation of proteins involved in Ca^{2+} handling and energetics. Intriguingly, many of the proteins showing increased S-nitrosylation with cardioprotection are mitochondrial proteins. It is likely that the high level of reactive cysteines in mitochondrial proteins, and the increased stability of N_2O_3 in the hydrophobic milieu of the mitochondria, would favor S-nitrosylation (21). Interestingly, although PostC and late-preconditioning both require $NO^{\bullet}$, protein S-nitrosylation has not been deeply examined under these conditions. We have shown that PostC induces an increase in S-nitrosylation of proteins, particularly low molecular weight proteins (150). In a recent study, addition of a mitochondria-targeted S-nitrosothiol at the beginning of reperfusion has also been found to be cardioprotective against ischemia/reperfusion injury (154). Sun *et al.* (179) also showed that perfusion of hearts with GSNO improved left ventricular function and protected against ischemia. Among others, mitochondrial F_1F_0ATPase-α-subunit and α-ketoglutarate-dehydrogenase were S-nitrosylated, and their activities were inhibited and enhanced, respectively. As said above, these results are in agreement with early studies, indicating that PreC saves post-ischemic ATP consumption, and protects against the loss of α-ketoglutarate activity (145, 201).

The cardioprotection by estrogen observed under conditions of increased contractility has been shown to involve an increase of S-nitrosylation of proteins. In fact, estrogen results in upregulation of cardiac eNOS and nNOS, and several S-nitrosylated proteins were identified, including the α-ketoglutarate dehydrogenase, the F_1F_0ATPase-α-subunit, the L-type calcium channel, and the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase, which reduces calcium loading during ischemia and early reperfusion, thereby reducing I/R injury (179). It has been suggested that atorvastatin- and sevoflurane-induced cardioprotection are mediated by increasing S-nitrosylation of cyclooxygenase-2 and sarcolemmal proteins, respectively (5, 18). Also the anti-apoptotic effect of $NO^{\bullet}$ has been linked to the inhibition of caspase-3 activation through cGMP-dependent and -independent mechanisms. In particular, S-nitrosylation–denitrosylation of caspase-3 were mechanisms by which Fas ligand was controlling the process of apoptosis (95, 114).

S-nitrosylated proteins could elicit their regulatory effects and protect cells by changing the structure and activity of proteins (*i.e.*, triggering signaling pathways), and/or protecting the modified cysteine residues from further irreversible modification under oxidative/nitrosative-stress, and/or

allowing the activity of these proteins/enzymes to be restored more quickly during I/R (Fig. 6) (179). It should be noted that, though nitrosylation and denitrosylation do not require enzymatic activities, they may be influenced by the presence of enzymes, metals and, of course, $NO^{\bullet}$ sources, thus determining compartmentalization (13, 114, 117).

S-sulfhydration

Hydrogen sulfide is a gas that can be physiologically generated from L-cysteine by the action of two enzymes cystathionine beta-synthase (CBS) and cystathionine gammalyase (CSE). Hydrogen sulfide physiologically modifies cysteines in a large number of proteins by S-sulfhydration, converting P–SH groups to persulfide group (P–SSH) (124), which thus appears to be another posttranslational modification for proteins (Fig. 5). Similar to nitrosylation the covalent modification in S-sulfhydration is reversed by reducing agents, such as DTT (124, 193). In the last few years, H_2S has been implicated in the regulation of several (patho)-physiological processes such as vasodilatation, cardioinotropism, and cardioprotection, to name just few. For instance, in $CSE^{-/-}$ mice, vessels and the heart do not form H_2S and animals show endothelial-dysfunction and hypertension (111); CSE inhibitors augment post-ischemic cardiac damage and H_2S donors are able to trigger cell signaling, leading to cardioprotection (47, 111). Importantly, both endogenous and exogenous H_2S induce PostC-like cardioprotection, involving the Akt–eNOS–PKC pathway. Moreover, the PKC inhibitor chelerythrine and the K_{ATP} channel blockers, glibenclamide or 5-hydroxydecanoate, are able to reverse the cardioprotective effects of the H_2S-donor, NaHS (89, 203). While it is not clear whether and/or how H_2S modifies the activity of RISK/SAFE enzymes involved in PostC, it seems that H_2S directly alters the activity of K_{ATP} channels. In fact, NaSH sulfhydrates the Kir6.1 subunit of K_{ATP} channels exogenously expressed in HEK293 cells. Treatment with DTT reverses the NaHS-mediated K_{ATP} sulfhydration (124). Moreover, H_2S interacts with Cys6 and Cys26 residues of the extracellular N-terminal of SUR1 subunit of K_{ATP} channel eterologhe complex. Direct chemical modification of SUR1 subunit protein may represent a molecular mechanism for the activation of K_{ATP} channels by H_2S (90). Yet at physiological concentrations, H_2S relaxes vascular tissues *via* the activation of sarcolemmal K_{ATP} channels in smooth muscle cells (193). Also the vasodilatation by garlic reflects H_2S action on K_{ATP} channels (111). Nevertheless, we should keep in mind that a direct K_{ATP} sulfhydration has not been demonstrated in the cardioprotective context. Moreover, the molecular basis of mK_{ATP} channels (in which cardioprotection seems more relevant than the sarcolemmal channels) remains unclear at present. Finally, it should be noted that cardiovascular K_{ATP} channels are generally thought to consist of Kir6.2/SUR2A subunits, in the case of heart muscle, or Kir6.1/SUR2B subunits in smooth muscle sarcolemma. However, there have been several reports of Kir6.1 mRNA and protein expression in the hearts of human, guinea pig, chick, rat, and mouse (103).

In the long run, the inducible form of heme oxygenase (HO), HO-1, may play a key role in maintaining antioxidant and oxidant homeostasis when cellular injury occurs (110). Notably, the H_2S donor, diallyl sulfide, induces the activation of ERK, the expression and nuclear translocation of the transcription factor Nrf2, and the activation of ho-1 gene. These results suggest that H_2S may play a role in prolonged protective effects by HO-1 overexpression (61). For additional data on the physiological role of H_2S, see reviews (111, 182).

Summary and Conclusions

Myocardial damage during reperfusion, among other processes, can be due to oxidative stress, the cellular/mitochondrial Ca^{2+} overload, the reduced availability of $NO^{\bullet}$, the recovery of intracellular pH, and the consequent formation of mPTP, leading to the massive liberation of ROS/RNS. The $NO^{\bullet}$ deficiency can also favor vasoconstriction, formation of micro-thrombi, adhesion of leucocytes to the endothelium, leading to the "no-reflow phenomenon" (1, 162, 169).

Cardioprotection by ischemic pre- and postconditioning shares some of the signal transduction pathways. In particular, both PreC and PostC studies suggest that early reperfusion acidosis is necessary to avoid mPTP opening and to allow ROS signaling (29, 30, 142). The protective cascades in early reperfusion converge on mitochondria and in particular on mPTP. In mitochondria, several proteins are phosphorylated. Beside kinase phosphorylation, other modalities of cell signaling are gaining ground. In particular, O-GlcNAcylation modification, S-nitrosylation, and S-sulfhydration of proteins are emerging as new signaling mechanisms that regulate cell function and play critical roles in mediating the response of cells to stress. It appears that when protein O- GlcNAcylation, S-nitrosylation, and S-sulfhydration increase, I/R injury is reduced, whereas when they decrease damage is exacerbated. However, we must keep in mind that the interplay between cell signaling may influence the outcomes. Cross-talk between different modalities of signaling may occur and may be the basis of controversial observations. Therefore a better understanding of this crosstalk may contribute to solve the conundrum relative to the role of RISK and/or SAFE pathway in cardioprotection deriving from conflicting results obtained with different models of pre- and postconditioning (14, 70, 131, 174, 190). It will be important to understand how post-transitional modifications of different proteins are regulated in the context of cardioprotection. For instance, it is likely that S-nitrosylation of different protein controls does not solely vary in function of the amount of $NO^{\bullet}$. Data suggest that besides kinases, phosphatates, antioxidant enzymes, and NOSs, other enzymes such those involved in O-GlcNAcylation (OGA and OGT) and/or in H_2S synthesis (CBS and CSE) can modulate post-ischemic cardiomyocyte survival. Subcellular localization of enzymes may favor or contrast protein post-transitional modifications and this likely contributes to the differences in final outcome.

Mitochondria play a role of paramount importance in ROS/RNS signaling and in cardioprotection and many of the considered post-translational processes also occur in these organelles. Understanding the compartmentalized subcellular signaling and crosstalk among them is the challenge researchers should face in the future, to better fight ischemia/reperfusion injury, especially in the presence of other comorbidities and therapies.

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Abbreviations Used

Akt = protein kinase B
ANT = adenine nucleotide translocase
CBS = Cystathionine beta-synthase
cGMP = cyclic GMP
CSE = cystathionine gamma-lyase
DTT = dithiothreitol
GPCR = G-protein coupled receptor
GSK-3β = glycogen synthase kinase
H_2O_2 = hydrogen peroxide
I/R = ischemia/reperfusion
IMS = mitochondrial intermembrane space
JAK = Janus activated kinase
MIM = mitochondrial inner membrane
mKATP = mitochondrial K^+ATP
MPG = N-(2-mercaptopropionyl)-glycine
mPTP = mitochondrial permeability transition pore
NAC = N-acetylcysteine
$NO^{\cdot}$ = nitric oxide
NOS = nitric oxide synthase
$O_2^{-\bullet}$ = superoxide anion
OGA = O-GlcNac transferase
$OH^{\bullet}$ = hydroxyl radical
OMM = outer mitochondrial membrane
$ONOO^-$ = peroxynitrite
P13K = phosphatidylinositol 3-kinase
p70S6K = P70s6 ribosomal protein S6 kinase
PKC = protein kinase C
PKG = protein kinase G
PostC = postconditioning
PreC = preconditioning
RACKs = receptors for active kinases
RICKs = receptors for inactive kinases
RISK = reperfusion injury salvage kinases
RNS = reactive nitrogen species
ROS = reactive oxygen species
S1P = sphingosine-1-phosphate
SAFE = survivor activating factor enhancement
SOD = superoxide dismutase
STAT3 = signal transducer and activator of transcription 3
VDAC = voltage-dependent anion channel