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Forum Review

Mitochondria as a Target for the Cardioprotective Effects of Nitric Oxide in Ischemia–Reperfusion Injury

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ABSTRACT

During cardiac ischemia–reperfusion (IR) injury, excessive generation of reactive oxygen species (ROS) and overload of Ca^{2+} at the mitochondrial level both lead to opening of the mitochondrial permeability transition (PT) pore on reperfusion. This can result in the depletion of ATP, irreversible oxidation of proteins, lipids, and DNA within the cardiomyocyte, and can trigger cell-death pathways. In contrast, mitochondria are also implicated in the cardioprotective signaling processes of ischemic preconditioning (IPC), to prevent IR-related pathology. Nitric oxide ($NO^{\cdot}$) has emerged as a potent effector molecule for a variety of cardioprotective strategies, including IPC. Whereas $NO^{\cdot}$ is most noted for its activation of the "classic" soluble guanylate cyclase (sGC) signaling pathway, emerging evidence indicates that $NO^{\cdot}$ can directly act on mitochondria, independent of the sGC pathway, affording acute cardioprotection against IR injury. These direct effects of $NO^{\cdot}$ on mitochondria are the focus of this review. *Antioxid. Redox Signal.* 10, 579–599.

INTRODUCTION

Nitric oxide ($NO^{\cdot}$) is a molecule with dichotomous activities in cardiac ischemia–reperfusion (IR) injury. Many reports correlate the production of $NO^{\cdot}$ to the oxidative stress and cellular damage seen in IR injury. Conversely, the cardioprotective effects of $NO^{\cdot}$ have been known since the late 1800s, when $NO^{\cdot}$ derivatives such as nitroglycerin were first used to treat angina. The divergent effects of $NO^{\cdot}$ in IR injury are based on the broad spectrum of $NO^{\cdot}$ biochemistry involved in cell death and cardioprotective pathways (129). Mitochondrial integrity correlates with IR injury, wherein lower myocardial recovery is seen under conditions in which ATP production becomes compromised and mitochondria become susceptible to permeability transition (PT) pore opening (123). Several mechanisms exist by which $NO^{\cdot}$ can act directly and indirectly on the mitochondrion to preserve bioenergetic integrity throughout IR injury. This review focuses on how $NO^{\cdot}$ directly regulates mitochondrial function, and the cardioprotective effects of this regulation. Although a primary focus of this article is on the protection elicited by ischemic preconditioning (IPC), it should be noted that several of the signaling mechanisms to be discussed are also applicable to other forms of preconditioning, including that elicited by volatile anesthetics (149, 196, 256) and several agents depicted in Fig. 1.

MITOCHONDRIAL ROS, Ca^{2+} HANDLING AND REDOX STATUS

Mitochondrial ATP generation, redox signaling (128), Ca^{2+} handling (35, 94), and cell-death regulation (35, 111) are important in maintaining cellular homeostasis and myocardial integrity. In synthesizing ATP, reducing equivalents (NADH and $FADH_2$) are generated during the oxidative breakdown of substrates, primarily fatty acids in the heart, to shuttle electrons into the electron-transport chain (ETC). The electrons are transferred from one respiratory complex to another down an electrochemical gradient to provide the energy needed to pump pro-

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tons from the mitochondrial matrix into the intermembrane space. This proton gradient is then used by the ATP synthase (complex V) to generate ATP.

Mitochondria are a major source of reactive oxygen species (ROS), from sites within the ETC (117, 128, 167, 230) and the Krebs cycle (231). The level of ROS generation ($O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$, *etc.*) defines the myocardial and mitochondrial redox environment. Overproduction of ROS, as seen after a prolonged ischemic insult, leads to oxidative stress and mitochondrial dysfunction by irreversibly oxidizing proteins, DNA, and lipids (128). However, lower levels of ROS production are known to trigger cardioprotective signaling cascades that preserve mitochondrial integrity (160) and the myocardium throughout IR injury.

Mitochondrial Ca^{2+} handling [for review, see (94)] is controlled by influx and efflux pathways. The influx pathways consist of the mitochondrial Ca^{2+} uniporter (MCU) and rapid-mode uptake (RaM), as well as a putative mitochondrially localized ryanodine receptor (mRyR) (19). Although the latter is believed to consist of the type-1 sarcoplasmic RyR (19), the protein identities of the MCU and RaM have not been identified. Influx of Ca^{2+} into mitochondria is controlled by the membrane potential ($\Delta\Psi_m$). Mitochondrial Ca^{2+} efflux involves both Na^+-dependent and independent pathways, with the former also dependent on $\Delta\Psi_m$. Some debate exists as to whether the PT pore (a nonselective channel in the mitochondrial membrane) may also serve as a Ca^{2+} efflux mechanism, either in its fully open mode more normally associated with pathology (see later) or in a transient "flickering" conformation reported to exist under cardioprotective conditions (108). However, efflux of Ca^{2+} through the PT pore appears unlikely under physiologic conditions, because such Ca^{2+} efflux would be dependent on passive diffusion ($[Ca^{2+}]_{mito}$ would have to be higher than $[Ca^{2+}]_{cytosol}$). Additionally, in energetically active tissues such as the heart, loss of $\Delta\Psi_m$ due to PT pore opening would be a high price to pay for achieving Ca^{2+} efflux.

At the mitochondrial level, overproduction of ROS and Ca^{2+} overload are known to trigger the pathologic opening of the PT pore and cell-death cascades. Opening of the PT pore results in mitochondrial swelling because of the equilibration of solutes between the mitochondrial matrix and the cytosol. This swelling in turn results in the rupture of the mitochondrial outer membranes and the release of proapoptotic factors such as cytochrome *c* and apoptosis-inducing factor (AIF), to initiate ATP-dependent apoptosis. Although a link between PT pore opening and cytochrome *c* release was discovered more than a decade ago, the precise mechanistic relation between these two phenomena remains unclear (245), and the requirement for mitochondrial swelling in this process is far from certain. In the absence of ATP, cells undergo necrosis as an alternative death cascade (99, 100), which has been associated with numerous pathologies, including IR injury.

MITOCHONDRIAL DYSFUNCTION IN IR INJURY

Several model systems of tissue damage that are relevant to cardiac IR injury have been used to study mitochondrial dysfunction. This includes hypoxia–reoxygenation, anoxia–reoxygenation, ischemia, ischemia–reperfusion, and autolysis. Although the experimental details of these models are different, they share many common features of mitochondrial pathology and are therefore discussed together under the global title "IR injury."

Hallmarks of cardiac IR injury are found to occur on reperfusion of the myocardium. In terms of the mitochondria, reperfusion injury affects the oxidative phosphorylation (Ox-Phos) pathway including the ETC (105, 106, 156, 187), adenine nucleotide translocase (ANT) (9, 214), and Krebs cycle enzymes (163, 202). In addition to Ox-Phos, reperfusion injury also leads to cardiolipin oxidation (155, 157, 187), the induction of a large proton leak across the mitochondrial inner membrane (22, 175), Ca^{2+} overload, overproduction of ROS, PT pore opening, and cell death (22, 29, 70, 73, 86, 99, 105, 106, 174, 219, 220, 230, 236). A majority of these observations have been made in mitochondria isolated from hearts after reperfusion, meaning that the time course of mitochondrial damage during reperfusion injury is difficult to study (because mitochondrial isolation typically takes 1–2 h). The exact timing of mitochondrial damage during IR is an ongoing subject of investigation.

Despite evidence for mitochondrial dysfunction occurring on reperfusion, the degree of dysfunction also depends on the length of the ischemic insult, suggesting that ischemia itself is detrimental. Accumulation of Ca^{2+} and ROS generation does occur within the mitochondrion during ischemia, despite mitochondria being de-energized (48). These events are linked to ischemic hypercontracture and the reversal of mitochondrial ATP synthase to use glycolytic ATP to maintain $\Delta\Psi_m$. Thus, a reconciling paradigm is that the degree of mitochondrial dysfunction during ischemia, which is a function of the length of ischemia, is a harbinger of more-severe mitochondrial dysfunction on reperfusion. In other words, more severe dysregulation of mitochondria and contractile machinery during ischemia (indicated by hypercontracture) leads to more-severe pathology on reperfusion.

MITOCHONDRIAL ROLE IN CARDIOPROTECTION

In contrast to the detrimental effects of prolonged ischemia, brief periods of ischemia initiate cardioprotective signaling cascades that preserve both myocardial and mitochondrial function during subsequent prolonged ischemia. This endogenous cardioprotective event was discovered more than 2 decades ago and is known as ischemic preconditioning (IPC) (173). Two "windows" of protection are elicited by IPC: the first acute phase is triggered within minutes and lasts 2–3 h, and a later delayed protective phase takes ~24 h to develop and lasts up to 72 h. This review primarily focuses on acute IPC.

Figure 1 shows a number of the signaling pathways implicated in IPC. Several well-known and emergent therapeutics also trigger the same pathways, including pharmacologic preconditioning (PPC) agents [*e.g.*, opioids, adenosine mimetics (256)], anesthetic preconditioning (APC) with volatile anesthetics (149), and physical interventions such as slow or intermittent reperfusion (ischemic postconditioning) (263). Thus,

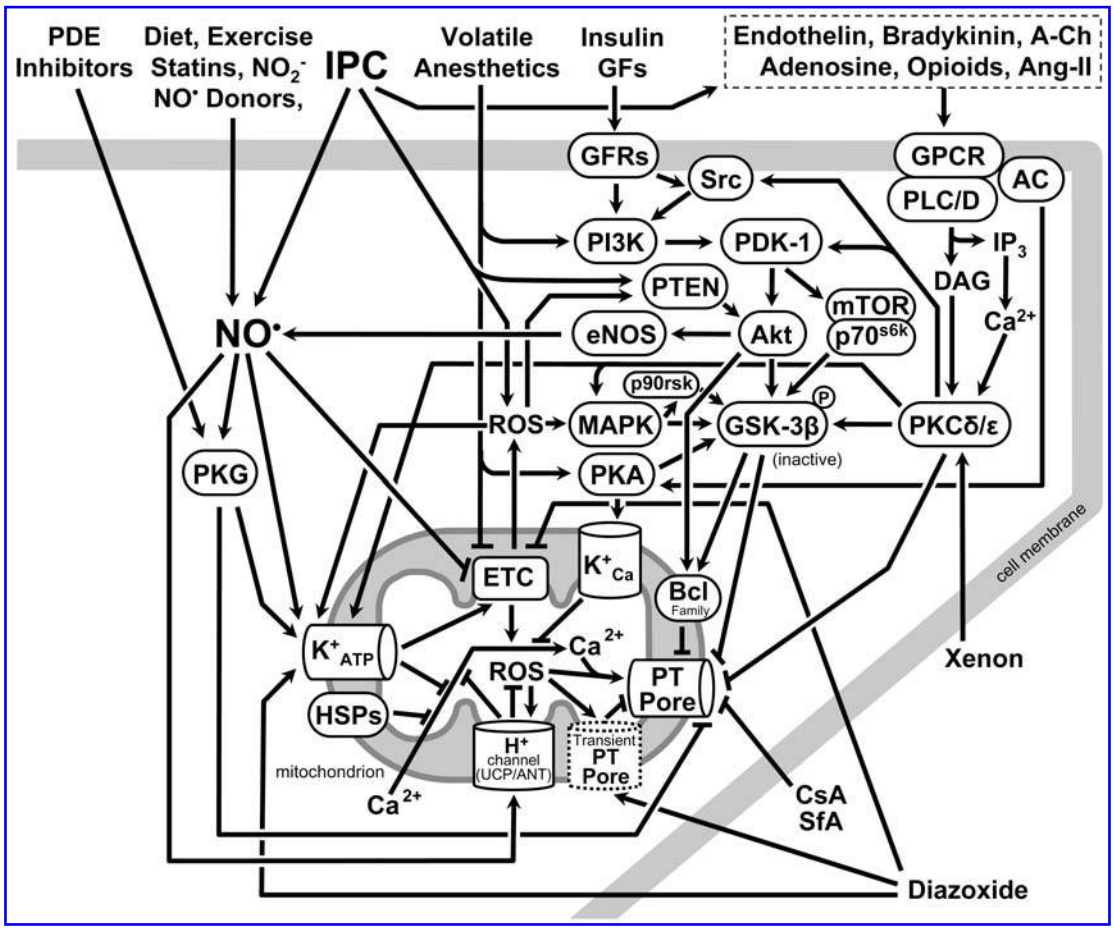

FIG. 1. Signaling pathways in IPC. The three main signaling pathways implicated in the mechanism of IPC are the insulin → PI3k kinase → Akt axis (110), the GPCR → DAG → PKCε axis (12, 65, 136, 181, 254), and the $NO^\cdot$ → PKG → K^+_{ATP} channel axis (60, 67, 85, 144). Key convergence points at the mitochondrial level are the phosphorylation and inactivation of GSK-3β (135), ROS generation by the ETC (10, 240), K^+_{ATP} channel opening (87, 92, 122, 162), and the inhibition of PT pore opening (100, 112). Roles are also proposed for $NO^\cdot$, mitochondrial uncoupling (H^+ leak) (175), transient opening or "flickering" of the PT pore (108), heat-shock proteins (137), K^+_{Ca} channels (221), and PTEN (45). The pathways by which various protective agents elicit cardioprotection also are shown (64, 76, 181, 247, 248). *Arrows*, Positive or stimulatory effects; *T-bars*, inhibitory effects.

x-PC signaling continues to be an area of interest, not only because it can help to explain how these therapeutic molecules work, but also because it provides a deeper understanding of preconditioning signaling pathways, which offers the hope of improved therapeutics to be developed in the future.

While the spectrum of available cardioprotective strategies continues to grow, one concept that emerges from Fig. 1 is that mitochondria are the downstream targets of most if not all of x-PC signaling. Key mitochondrial events include mild uncoupling (H^+ leak), opening of mitochondrial K^+_{ATP} channels, inhibition of mitochondrial Ca^{2+} overload, attenuation of ROS generation, and inhibition of PT pore opening at reperfusion (108, 109, 123, 129). Notably, several of the mitochondrial events associated with IR injury are thought to occur in a limited manner during IPC. For example, a somewhat paradoxic situation is proposed wherein transient "flickering" of the PT pore during the triggering/initiation phase of IPC is thought subsequently to prevent large-scale PT pore opening during IR injury (108, 109). In addition, limited ROS generation has been found to be essential in IPC signaling cascades, and the cardioprotective response was found to be blocked by antioxidants (225). Similar to PT pore opening and ROS production, a large increase in H^+ leak is found to occur in IR injury (affecting mitochondrial ATP synthetic capacity). However, a small reversible increase in H^+ leak is seen during IPC, which may act to diminish ROS generation and Ca^{2+} overload at reperfusion (175). Pharmacologic preconditioning agents, such as diazoxide (K^+_{ATP} channel agonist) and dinitrophenol (DNP, induces mitochondrial proton leak), also elicit cardioprotection in IR injury by attenuating mitochondrial PT pore formation, ROS generation, and H^+ leak (108, 169, 185).

The overarching principle of x-PC signaling can be summarized in the proverb "what doesn't kill you makes you stronger." It is therefore important to note that this principle is played out in its entirety in the microcosm of the mitochondrion. Whereas Fig. 1 highlights a multitude of upstream signaling pathways converging on mitochondria to elicit cardioprotection, a full discussion of these mechanisms is beyond the scope of this re-

view. Therefore, the remainder of this article focuses on the pathways affected by nitric oxide ($NO^{\cdot}$).

$NO^{\cdot}$ BIOCHEMISTRY AND SIGNALING

A second important paradigm emerging from Fig. 1 is the critical role of $NO^{\cdot}$ in various cardioprotective signaling pathways. This is discussed in more detail later, after a discussion of NO biochemistry and signaling in general and the controversial role of $NO^{\cdot}$ in IR injury. The wide range of $NO^{\cdot}$ signaling arises from $NO^{\cdot}$ being a freely diffusible gas and having diverse biochemistry, which leads to the formation of multiple secondary intermediates, such as $NO^{\cdot}$, NO^+, NO^- (HNO), $ONOO^-$, NO_2^-, and $NO_2^{\cdot}$. Collectively, these species are referred to as reactive nitrogen species (RNS), or sometimes as "NO" (without the radical dot). Each of these intermediates can participate to varying degrees in the modification of biomolecules:

Protein modification

NO-dependent protein modification can be divided into nitrosylation, nitrosation, and nitration reactions (Fig. 2A). Nitrosylation involves $NO^{\cdot}$ interaction with heme and metal centers, as seen in sGC activation to initiate cGMP-dependent signaling (129). Similarly, mitochondrial complex IV (cytochrome *c* oxidase) is regulated by the formation of a nitrosyl-cytochome a_3 complex (36). Nitrosation involves the modification of oxygen (O-nitrosation), nitrogen (N-nitrosation), or sulfur (S-nitrosation) within amino acids (113). S-nitrosation of protein thiols has been observed *in vivo* and is associated with physiologic protein regulation and cellular signaling (17, 55, 115, 124, 125, 251, 255). Both N-nitrosation and O-nitrosation reactions are typically seen only with high $NO^{\cdot}$ exposure, are not as readily reversible, and are more commonly designated as markers of oxidative or nitrosative stress (113). Because of its irreversibility, nitration of tyrosine residues (formation of 3-nitrotyrosine) has also been traditionally viewed as a marker of oxidative stress resulting from $ONOO^-$ and its radical products ($NO_2^{\cdot}$, $OH^{\cdot}$) (4, 192, 193). The generation of $ONOO^-$ leads to the nitration of proteins such as MnSOD (252), which is thought to compromise mitochondrial integrity. Doubt has been cast over defining N-Tyr as a finger print for $ONOO^-$ generation, because N-Tyr formation has also been shown to be driven by myeloperoxidase (MPO) reactions. In addition, the irreversibility of Tyr nitration has also been questioned, since the discovery of potential "denitrase" enzymes (146). Nitrosylation, nitration, and nitrosation at the mitochondrial level are known to regulate respiration (30, 34, 174), Ca^{2+} handling (72), H^+ leak (29, 31), and ROS generation (174, 189). The implications of NO-dependent regulation of these mitochondrial functions throughout IR is discussed in detail later.

Lipid modification

Reactive nitrogen species can serve as either oxidants or antioxidants depending on the conditions in the lipid environment. Several RNS (most notably $ONOO^-$) are known to contribute to oxidative stress by inducing lipid oxidation chain reactions under conditions in which ROS and $NO^{\cdot}$ are being produced (200). However, $NO^{\cdot}$ can also serve as a highly lipid-soluble chain-terminating antioxidant in lipid-oxidation cascades, when present at higher amounts than ROS (119, 200). Among the

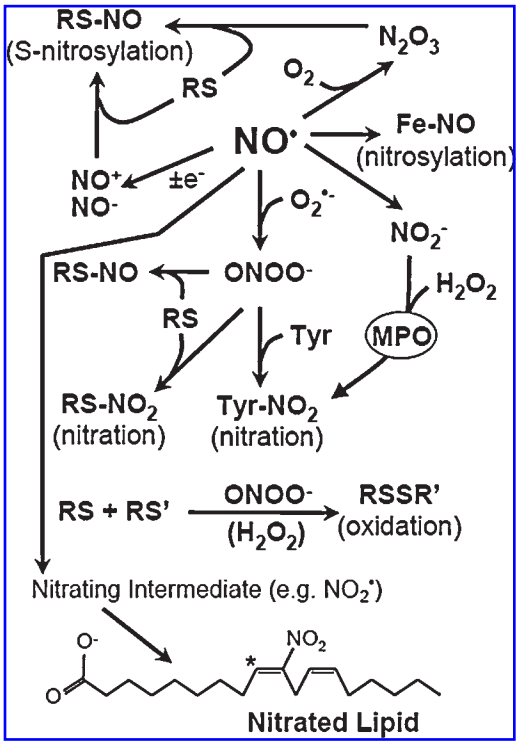

FIG. 2. $NO^{\cdot}$-dependent modification of biomolecules. Nitric oxide can noncovalently bind to heme-iron to yield a nitrosyl-heme (Fe-NO). In addition, reaction with O_2 can yield RNS, which can oxidize proteins by forming disulfides (RSSR), nitrosation (addition of NO), or nitration (addition of NO_2) of proteins. For example, $NO^{\cdot}$ reacts rapidly with $O_2^{\cdot-}$, forming peroxynitrite ($ONOO^-$) that leads to protein-oxidation reactions including nitrosation (197). It is also known that myeloperoxidase (MPO) can mediate nitration reactions using nitrite (NO_2^-) and H_2O_2 as substrates (239). Overall, oxidation reactions (*e.g.*, oxidation of glutathione) occur at the highest chemical yield, but nitration (*e.g.*, formation of nitrotyrosine) or nitrosation (*e.g.*, formation of *S*-nitrosoglutathione) are also potent mediators of biological responses (14). The nitrogen monoxide radical ($NO^{\cdot}$) can gain/lose an electron, yielding the nitroxyl anion (NO^-/HNO) or nitrosonium cation (NO^+), respectively. Both NO^- and NO^+ have unique bioactivity relative to $NO^{\cdot}$. For example, NO^+ is proposed to form *S*-nitrosothiols by reaction with the thiolate anion (RS^-). *Via* intermediates that are not fully understood, $NO^{\cdot}$ can also lead to the generation of nitrated lipids such as nitro-oleate and nitro-linoleate (shown) (13). Nitro-lipids feature an electrophilic carbon center (*), which makes possible the adduction of these species onto nucleophiles (*e.g.*, protein thiols). Other NO-dependent reactions described in this review include the Fe-NO complexes that form at low concentrations with cytochrome *c* oxidase (38, 57, 204), and the *S*-nitrosothiols and nitrotyrosine that form within mitochondrial enzymes such as complex I (58, 168, 201).

newest findings in the field of RNS research is the recent discovery of biologically active nitrated lipids (LNO_2) at endogenous micromolar levels in human tissues and plasma (13 183). Nitro-lipids such as nitro-linoleate and nitro-oleate (Fig. 2B) can be formed by several mechanisms, including (a) reaction of $NO^{\cdot}$ or RNS with lipid oxidation products; (b) at low pH, the reaction of NO_2^- with unsaturated lipids or reaction of nitrous acid with oxidized lipids; and (c) at physiologic pH, the reaction of $NO_2^{\cdot}$ with unsaturated lipids. Because mitochondrial membranes are rich in polyunsaturated lipids (71, 187), it is possible that endogenous generation of LNO_2 within mitochondria may occur during ischemia, because of a decrease in pH, and $NO^{\cdot}$ levels are elevated. The effects of LNO_2 on mitochondria have not been defined but are thought to have a significant impact in regulating this membranous organelle.

DNA

Although $NO^{\cdot}$ itself is not widely recognized as a DNA-"damaging" species, $ONOO^-$ can oxidize several DNA bases. Most notably, $ONOO^-$ directly activates the DNA-repair enzyme PARP (226), which depletes NAD^+ levels in the cell, raising the possibility that $ONOO^-$-induced PARP activation contributes to the depletion of pyridine nucleotides that occurs in IR injury. Interestingly, it has recently been shown that the PARP inhibitor PJ-34 preserved NAD^+ levels and was protective in a cardiomyocyte model of IR injury (80).

When considering the actions of a diverse signaling molecule such as $NO^{\cdot}$ (Fig. 2) in the complicated signaling milieu encountered in IR or x-PC, it is clear that consideration must also be given to a host of other factors that can affect $NO^{\cdot}$ biochemistry and signaling. One notable example is pH, with several RNS having pK_a values at or close to 7 (*e.g.*, $ONOO^-$/ONOOH couple pK_a is 6.8). Another is O_2 concentration, with several RNS biochemical reactions being dependent on the presence or absence of O_2. A third is microenvironment, in which local concentrations of species such as glutathione (GSH; *e.g.*, in mitochondria), or heme (*e.g.*, myoglobin in the cardiomyocyte) can have a huge impact on $NO^{\cdot}$ signaling. Consideration of the biochemistry of $NO^{\cdot}$ yields a picture of an extremely specific set of signaling pathways, resulting in targeted actions of a seemingly nonspecific free radical (129, 158).

THE DIVERGENT ROLES OF $NO^{\cdot}$-IR INJURY

When considering the roles of NO in any cell-signaling process, it is important to discuss the sources of NO generation. Nitric oxide synthase (NOS) enzymes produce $NO^{\cdot}$ endogenously and are regulated by many of the upstream signaling pathways that are activated during IR and x-PC [Fig. 1; for review see (3)]. Studies on the cardioprotective and deleterious effects of $NO^{\cdot}$ have involved manipulation of endothelial NOS (eNOS) and inducible NOS (iNOS) activity. Attenuation of myocardial infarct size and preservation of left ventricular developed pressure and lower left ventricular end-diastolic pressure have been observed in mice overexpressing eNOS in a variety of models of ischemia-alone or IR injury, when compared with wild-type mice (39, 132, 133). The NOS-dependent protection was found to be abrogated with the administration of a commonly used NOS inhibitor, *N*(G)-nitro-L-arginine methyl ester (L-NAME) (39 132). In agreement, studies using eNOS knockout mice have found an augmentation in infarct size (131) and myocardial necrosis (209) after IR injury. In terms of timing, eNOS is activated during the early window of preconditioning and activates transcription factors and other enzymes, including iNOS, in the late window of protection (129). The activation of eNOS has also been reported to contribute to the cardioprotective effects of postconditioning (brief intermittent reperfusion periods after ischemia). As seen in the eNOS transgenic and knockout studies, the cardioprotective effect of postconditioning was sensitive to L-NAME (129, 234). Furthermore, support for the essential role NOS plays in cardioprotection is seen with the sexual dimorphism in responses to IR injury; estrogen has been found to increase NOS expression, which may help explain why premenopausal women possess a lower risk of heart disease when compared with age-matched men (66).

Several studies suggest the presence of a mitochondrial isoform of NOS (mtNOS), although the reproducibility of these studies appears to be limited to a small number of laboratories (88, 140, 238). Several recent articles have questioned the existence of mtNOS, with major controversies surrounding the purity of mitochondria, a 150,000-fold variation in the reported rates of NO generation, and several experimental artifacts in NO-measurement systems (28, 150). It was reported that a NOS protein in the plant *Arabidopsis thaliana* (atNOS) is targeted to the mitochondrion, and more recently a mammalian orthologue of this protein was proposed as a candidate for mtNOS (96, 260). However, further investigation has found that the protein is a GTPase, not an NOS (63, 95, 259). Thus, the search for a unique mtNOS protein continues.

NOS enzymes have also been shown to play a deleterious role in IR injury. For example, $iNOS^{-/-}$ mice were shown to have lower mortality and enhanced left ventricular contractility when compared with wild-type mice after coronary occlusion (79). Also, exposing mitochondria to high concentrations of $NO^{\cdot}$ (micromolar) has been shown to initiate PT pore opening (33). These results, along with other studies, have defined $NO^{\cdot}$ as a dual-faced molecule in IR injury, which contributes to both cardioprotective and deleterious signaling pathways within the myocardium. In this regard, understanding how to deliver $NO^{\cdot}$ (*i.e.,* timing, concentration, location) may facilitate beneficial therapeutic exploitation of $NO^{\cdot}$ signaling in IR injury, while minimizing the deleterious effects of $NO^{\cdot}$. In the following sections, the specific cardioprotective actions of $NO^{\cdot}$ are discussed in more detail.

THE DIVERGENT ROLES OF NO: CARDIOPROTECTION *VIA* "CLASSIC" $NO^{\cdot}$ SIGNALING

The cardioprotective effects of $NO^{\cdot}$ during IR include vasodilatation, inhibition of platelet aggregation, antiinflamma-

tory responses, and antiapoptotic processes (126, 129, 201). The "classic" $NO^{\cdot}$ signaling pathway (*i.e.,* binding of $NO^{\cdot}$ to sGC, generation of cGMP, activation of protein kinase G) has been heavily implicated in the process of NO-mediated cardioprotection (148, 201). However, the sGC/cGMP pathway is not the only cardioprotective response triggered by $NO^{\cdot}$.

As highlighted in Fig. 1, several cardioprotective pathways appear to converge on both NO and the mitochondrion to elicit cardioprotection. Some mitochondrial targets of the classic cGMP/PKG signaling pathway have also been identified, including the opening of the mitochondrial K^{+}_{ATP} channel (60), the triggering of mitochondrial biogenesis *via* the activation of PGC-1α (178), the expression of mitochondrial redox-regulatory proteins (thioredoxin, HSP70, MnSOD) (53, 54), and the inhibition of the PT pore (144). Several issues remain to be resolved with such signaling pathways, most notably the question of how a PKG phosphorylation signal crosses the mitochondrial membranes (191). The details of the downstream $NO^{\cdot}$ signals that mediate cardioprotective responses require further definition; we discuss the mitochondrion as a target for NO-dependent cardioprotection. In particular, focus is given to nonclassic NO signaling pathways and those involving the direct interaction of NO species on the mitochondrion during preconditioning.

$NO^{\cdot}$ AND MITOCHONDRIA-DEPENDENT CARDIOPROTECTION

Increasing evidence indicates that NO is a key mitochondrial regulator; for a number of reasons, the mitochondrion can be considered a cellular "hub" for NO signaling. First, mitochondria within the cardiomyocyte are in close proximity (1–2 μm) to the production site of $NO^{\cdot}$ (194, 233). Second, $NO^{\cdot}$ is freely diffusible and partitions into membranous environments such as mitochondria, which contribute ~30% of the typical cardiomyocyte volume (32). Last, mitochondria are enriched in metal centers and thiols, and they generate ROS that interact with $NO^{\cdot}$ to produce a number of secondary intermediates important for NO signaling (Fig. 2).

Before describing the protective effects of NO signaling, it is important briefly to discuss the deleterious side of NO at the mitochondrial level. Many of the deleterious effects can be attributed to the overproduction of $NO^{\cdot}$ by iNOS or administration of NO donors at high concentrations, which cause irreversible oxidation of proteins, lipids, and DNA. Indeed, well before the identity of endothelium-derived relaxing factor was known, it was discovered that macrophages were able to generate a species (now identified as $NO^{\cdot}$) that could inhibit cellular respiration (91). In contrast, lower endogenous production of $NO^{\cdot}$ and smaller concentrations of NO donors have been found to protect mitochondria during situations such as IR injury. An example of this is the dose–response dependence of isolated mitochondria to PT pore opening with $NO^{\cdot}$ treatments (33). High levels of $NO^{\cdot}$ (>5 μM) were found to induce PT pore formation, whereas lower levels of $NO^{\cdot}$ (100 nM to 1 μM) were found to inhibit pore opening. The following effects of NO on mitochondrial function are defined predominately in terms of cardioprotection, because they are compared with x-PC agents that induce NO-independent cardioprotection (Fig. 3).

Electron transport chain (overview)

During ischemia/hypoxia/anoxia, the lack of O_2 causes a backup of electrons and reduction of cytochromes within the ETC. On reperfusion, these electrons are passed onto O_2 both *via* the conventional route at complex IV to generate H_2O and *via* complexes I and III to generate ROS. These ROS can either initiate cardioprotective signaling cascades, or, if produced at high levels, lead to oxidative stress. The cardioprotective effects of NO signaling on the ETC are thought to occur by (a) reversibly inhibiting electron entry into the ETC, (b) generating low levels of ROS to initiate cardioprotective cascades, and (c) inhibiting cytochrome *c* peroxidase activity.

Reversibly inhibiting proximal components of the ETC by NO is thought to result in the slow reintroduction of electrons into the ETC on reperfusion. Pharmacologic agents that reversibly inhibit complexes I and II are cardioprotective, and their response may be elicited by forcing the ETC to undergo a "gradual wakeup" from ischemia on reperfusion (42, 174). This gradual wakeup is thought to attenuate such events as mitochondrial Ca^{2+} overload, ROS overproduction, and PT pore formation (Fig. 4). However, a limited amount of ROS is needed to initiate preconditioning signaling pathways (160), which could be triggered by interactions of NO with distal components of the ETC, such as complex III and IV (Fig. 3). Nitric oxide also prevents oxidative damage to cardiolipin (241) by inhibiting cytochrome *c* peroxidase activity. Taken together, the ETC is a site of direct NO-dependent regulation and controls mitochondrial ROS generation, Ca^{2+} overload, and PT pore formation.

Complex I (NADH dehydrogenase)

This complex is a 46-subunit protein complex consisting of nine Fe-S centers, an FMN site, and numerous thiols. Electrons are passed from NADH though complex I onto coenzyme Q (CoQ), resulting in pumping of protons into the intermembrane space. In terms of IR injury, complex I is the first ETC enzyme to sustain damage (199) leading to an elevation of ROS production (237). A number of factors can contribute to elevated ROS generation from complex I, including thiol oxidation, Fe-S integrity, and electron buildup due to downstream ETC inhibition. Reversible inhibition of complex I by volatile anesthetics or other inhibitors has been found to be cardioprotective for both mitochondria and cardiac tissue. For example, treatment with the barbiturate amytal (2–2.5 mM) before ischemia preserves mitochondrial ETC function, cytochrome *c* content, and pyridine nucleotide levels (49, 237).

In terms of NO-dependent regulation, complex I is reversibly inhibited by S-nitrosation (21, 42, 52, 58, 68) (Fig. 3), and is S-nitrosated under cardioprotective conditions in both cardiomyocyte and perfused heart model systems (174). Studies from our laboratory identified the 75-kDa subunit of complex I to be a target of S-nitrosation, which is in agreement with other groups that have reported on the probability of this subunit being S-nitrosated based on amino acid sequence (52). Modification of this site inhibits complex I-linked NADH ox-

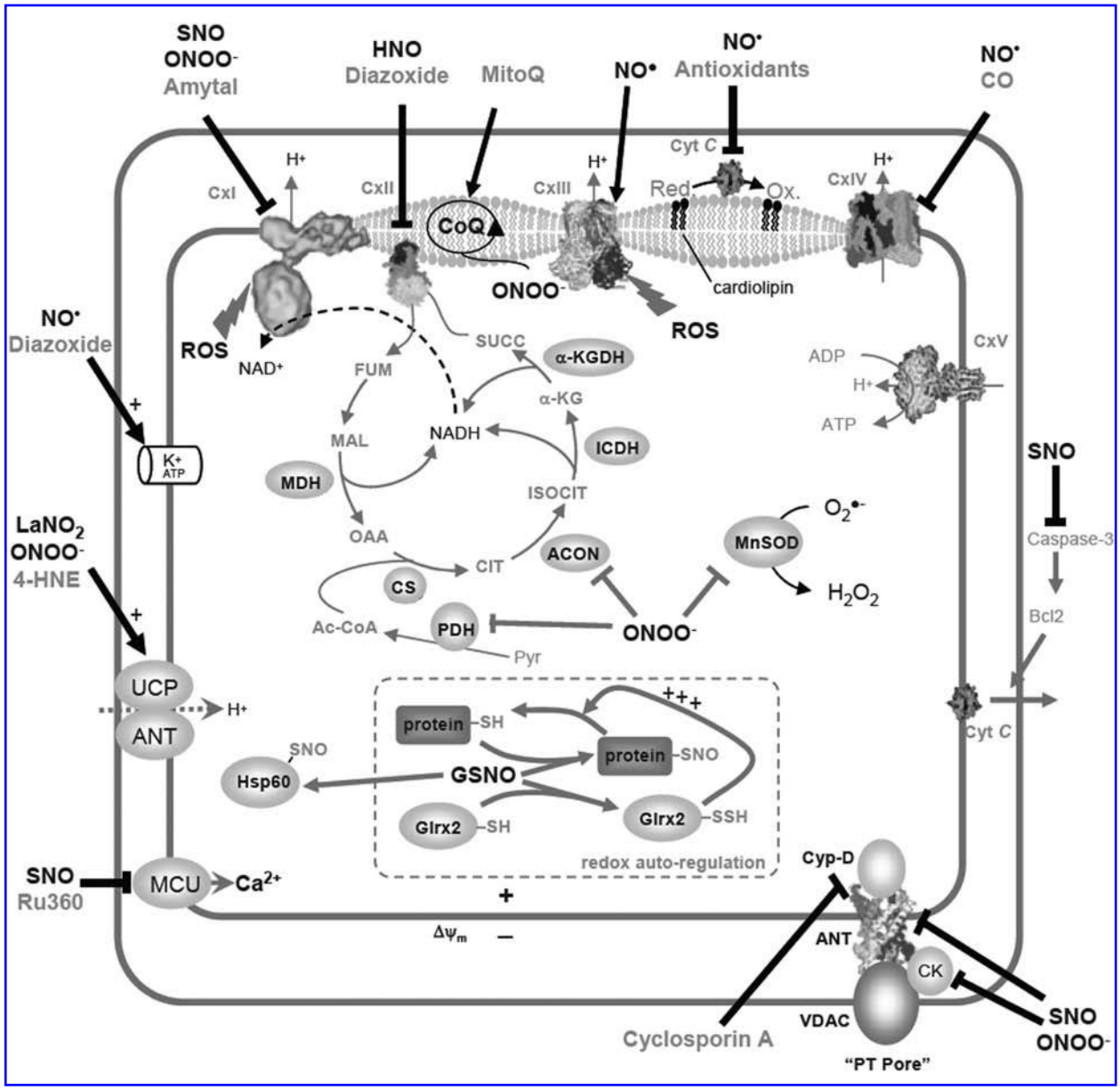

FIG. 3. NO targets in mitochondria with relevance to cardioprotective mechanisms. Several mitochondrial targets for the cardioprotective effects of NO are shown, including **ETC:** reversible inhibition. The ETC has been found to preserve mitochondria in IR injury. Nitric oxide–based inhibitors of complex I (SNO), complex II (HNO), and complex IV (NO^-) have been hypothesized to be cardioprotective (see text for details). Note: A truncated version of Krebs cycle is shown for clarity. **Ca^{2+}:** NO-mediated inhibition of mitochondrial Ca^{2+} influx may also provide cardioprotection in IR injury. Although identification of direct NO modification on mitochondrial Ca^{2+} has not been made, other cytosolic Ca^{2+} channels have been reported to be S-nitrosated (L-type Ca^{2+} channel, sarcoplasmic RyR; see text for details). Attenuation of ROS generation and inhibition of Ca^{2+} leads to the prevention of the **PT pore**. Although NO^- and other RNS have been reported to inhibit the PT pore, the direct target remains to be identified. Induction of a mild **H^+ leak**, *via* activation of uncoupling proteins, also protects the mitochondrion from the PT pore and IR injury. Administration of low levels of $ONOO^-$ triggers an H^+ leak. Nitrosylation of the heme in cytochrome *c* has been reported to inhibit oxidation of **cardiolipin** and release of cytochrome *c* to the cytosol. In addition to showing the effects of NO and other RNS, this figure highlights some of the pharmacologic agents that act on the same cardioprotective pathways. This includes amytal (complex I inhibitor), cyclosporin A (PT pore inhibitor), and Ru360 (Ca^{2+} uniporter inhibitor). Inset (*dotted-line box*): Proposed mechanism of redox regulation of S-nitrosation. Although the specific details of how mitochondrial SNO are metabolized is in need of further definition, studies have highlighted the importance of the mitochondrial redox regulatory system components such as glutaredoxin 2 (107) (Glrx2). Small RSNOs (*e.g.*, GSNO) *trans*-nitrosate proteins to generate a cardioprotection signal, but also *trans*-nitrosate redox enzymes (*e.g.*, Glrx2) needed for signal regulation, which then mediate the reversal of S-nitrosation on other proteins. Arrows, positive or stimulatory effects; T-bars, inhibitory effects.

idation and therefore entry of electrons into complex I and the ETC (42, 174). Notably, the degree of inhibition of complex I by S-nitrosation never increases above ~30–40%, which may be important in timely reversal of the modification and recovery throughout reperfusion. In addition to attenuating electron entry into the ETC, reversible inhibition of complex I may also affect the NADH/NAD^+ pool. Higher levels of NADH could be used to preserve the mitochondrial antioxidant machinery (glutathione and thioredoxin) (128), and NADH is known to regulate the PT pore potently.

Complex II (succinate dehydrogenase)

This complex is a component of the ETC and the Krebs cycle, which passes electrons from $FADH_2$ onto CoQ. The inhibition of complex II by 3-NP has been found to be cardioprotective by limiting ROS levels and reducing infarct size after reperfusion in a rat model of IR injury (235). In addition, diazoxide, a known cardioprotective agent (244), is known to inhibit complex II (206). Nitric oxide–dependent reactions involving complex II are not as defined as complex I S-nitrosation, although complex II was found to be reversibly inhibited in isolated mitochondria after administration of a nitroxyl (HNO) donor (212) (Fig. 3). It was found that HNO modified complex II thiols in a manner independent of S-nitrosation, and that this was reversible by glutathione. Limited evidence exists for the formation of HNO in the mitochondrion (44, 210), but it has been suggested to be a product of SNO breakdown (1, 7). Previously HNO was defined as a marker for NO cytotoxicity, but like several other RNS, is now being recognized as a cardioprotective agent (186). Further evidence is needed to determine whether HNO directly interacts with complex II under physiologic conditions.

Co-enzyme Q

Electrons are passed from complexes I and II to complex III, *via* CoQ. The reduced form of CoQ (ubiquinol) can function as an antioxidant and, based on reduction potentials, can react with NO species such as $NO^{\cdot}$, $NO_2^{\cdot}$, and $ONOO^-$ (190). During ischemia, ubiquinol concentrations would be elevated, and its antioxidant properties may serve an important role to scavenge the burst of ROS and RNS on reperfusion (Fig. 3). Therefore, ubiquinol can be thought of as a mechanism by which mitochondria regulate RNS concentrations, and this may be one mechanism by which mitochondrially targeted ubiquinol derivatives, such as MitoQ, afford cardioprotection in IR injury (127).

Complex III (ubiquinol cytochrome c oxidoreductase)

Complex III transfers electrons from ubiquinol to cytochrome *c* and is a site of ROS generation within the mitochondrion (117). It has been reported that $NO^{\cdot}$ binding to the bc_1 segment of complex III can result in elevated ROS generation (189) (Fig. 3). The level of ROS generated is proportional to the concentration of ubiquinol, in which the ROS produced by complex III could be viewed as a trigger for oxidative stress or the initiator for cardioprotective signaling needed for the second window of cardioprotection (189).

It should be noted, however, that the effect of NO on generation of ROS from the ETC is controversial and far from settled. Although it has been hypothesized (32) that NO can lead to an increase in mitochondrial ROS, data supporting this are sparse (189). Further doubt over this hypothesis is cast by the fact that inhibition of mitochondria at complex IV with CN^- does not increase ROS generation (43). In addition, although treatment of mitochondria with large doses of NO donors under nonphysiologic conditions (25°C) leads to increased ROS generation from complex I (21), we recently showed the opposite (*i.e.*, S-nitrosation of complex I leads to a decrease in ROS generation) (174). Therefore, further investigation is needed to

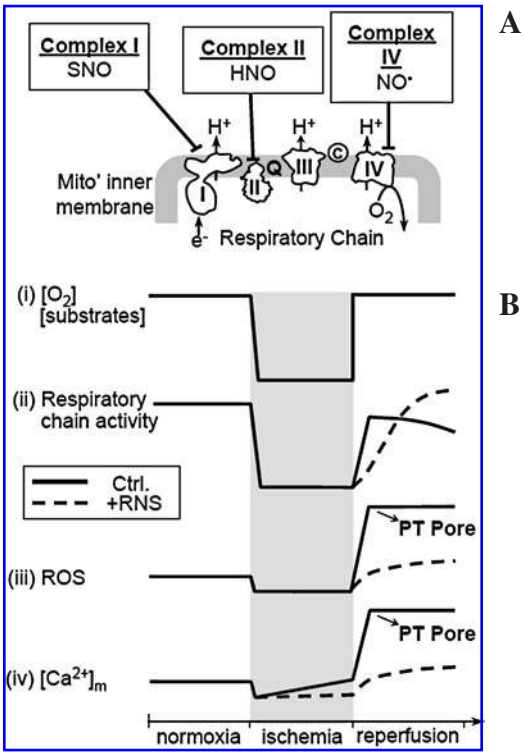

FIG. 4. Gradual wakeup hypothesis. (**A**) Protection against IR injury is afforded by administration of several respiratory-chain inhibitors, as detailed in the text. The respiratory complexes at which these inhibitors act are denoted by Roman numerals. The mitochondrial outer membrane is omitted for clarity. **(B)** Hypothetical mechanism by which reversible respiratory inhibition may protect against IR injury. Upper trace (i) shows profile of $[O_2]$ and [substrates] during IR; trace (ii) shows mitochondrial respiratory-chain activity; trace (iii) shows mitochondrial ROS generation; and trace (iv) shows mitochondrial Ca^{2+} uptake. Respiratory inhibition naturally occurs during ischemia because of lack of O_2, and under normal IR conditions (solid lines), reperfusion results in a rapid recovery of respiration and a surge in Ca^{2+} uptake and ROS generation. This leads to PT pore opening and Ca^{2+}/ROS-induced damage to Ox-Phos, inhibiting respiration later during reperfusion. With inhibitors present (dotted lines), the respiratory chain remains partially inhibited during the early reperfusion period, preventing Ca^{2+} overload, ROS generation, and PT pore opening. Subsequent inhibitor washout then permits full recovery of respiratory function.

determine if NO *in vivo* can trigger the generation of ROS needed to initiate preconditioning and other signaling pathways.

Cytochrome c/cardiolipin

Cytochrome *c* functions in the ETC to pass electrons from complex III to complex IV. Cardiolipin is a mitochondrial phospholipid that has been reported to be oxidized during IR (155, 157, 187). Oxidation of cardiolipin is linked with permeabilization of the outer membrane and jeopardizes the integrity of membrane-bound enzymes such as complex I (188). Recently, the oxidation of cardiolipin has been proposed to occur *via* a peroxidase activity adopted by cytochrome *c* (15). Under normal conditions, a hexa-coordinate arrangement is found around the heme iron of cytochrome *c*, and small molecules such as $NO^{\cdot}$, CO, O_2, and H_2O_2 cannot access the heme. However, loss of the axial ligand results in a shift to a penta-coordinate heme, to initiate peroxidase activity. Nitric oxide has been shown inhibit the peroxidase activity of the cardiolipin/cytochrome *c* complex *via* nitrosylation, and therefore protect the mitochondria from outer membrane permeabilization (Fig. 3), but further investigation is needed to determine whether this is an *in vivo* phenomenon (241). In addition, it is reported that nitrosylation of cytochrome *c* triggers its release from the inner membrane, which would appear to contradict such findings (207).

Complex IV (cytochrome c oxidase)

Probably the best characterized effect of $NO^{\cdot}$ on the mitochondrion is the reversible inhibition of complex IV. Complex IV is the terminal component of the ETC where electrons are passed on to O_2 *via* a binuclear heme–copper center (heme a_3 and Cu_B), to form H_2O. The heme–copper center can react with $NO^{\cdot}$ and be nitrosylated *via* two different mechanisms, which result in the release of $NO^{\cdot}$ or NO_2^-, depending on whether $NO^{\cdot}$ binds to a reduced or oxidized binuclear site (204). The mechanism of $NO^{\cdot}$-dependent complex IV inhibition has been found to be dependent on a number of factors that control the redox environment within the enzyme, including electron flux, O_2 tension, and $\Delta\Psi_m$ (20, 30, 34, 40, 89). Inhibition of complex IV by $NO^{\cdot}$ would elicit a cardioprotective response by preserving the limited O_2 supply to tissues farther away from the blood supply (229). In agreement, other molecules that bind to the binuclear site within complex IV, like carbon monoxide, are also cardioprotective (56) (Fig. 3).

Redox-regulatory and heat-shock proteins

The mitochondrial redox status is tightly regulated by a network of molecules and enzymes. This network has been found to be both irreversibly modified by RNS (*e.g.,* nitration of Mn-SOD) and regulated by reversible NO modifications, such as S-nitrosation. This section focuses on how the mitochondrial redox network regulates and is regulated by S-nitrosation to elicit cardioprotective responses.

Glutathione (GSH) has been reported as key regulator of mitochondrial S-nitrosation. Glutathione is the most abundant antioxidant within the cell and exists at local concentrations up to 10 m*M* (107). The mitochondrial pool of GSH has been reported to contribute to the preservation of protein thiols in the relatively "ROS-abundant" environment of the mitochondrion. In the presence of RNS, GSH is S-nitrosated, and *S*-nitroso-glutathione (GSNO) reacts with thiols to *trans*-nitrosate mitochondrial proteins (Fig. 3). It has also been proposed that in the presence of reduced glutathione, S-nitrosated peptides may become glutathionylated, another reversible thiol modification (52). *Trans*-nitrosation with other endogenous S-nitrosating agents, such as Cys-NO, can also occur inside the mitochondria. The transport of small *S*-nitrosothiols like Cys-NO has been found to be more efficient than GSNO (261).

A number of possible mechanisms have been reported to reverse *trans*-nitrosation reactions. One of the most recent findings in this field involves the regulation of thiols with the mitochondrial isoform of glutaredoxin (Glrx2). This enzyme is responsible for the reduction of disulfides and has been reported to reverse glutathionylation reactions (Fig. 3). Activation of Glrx2 has been observed in the presence of GSNO and the $ONOO^-$ donor Sin-1. However, under the same conditions, the cytosolic isoform Glrx1 was found to be irreversibly inhibited by both GSNO and $ONOO^-$ (107). This specific example highlights how mitochondria have evolved the ability to regulate SNO signaling. Other mitochondrial redox enzymes have been reported to be regulated by NO and to regulate NO-dependent signaling. Thus, preservation of their activity may serve as a switch point between NO-dependent cardioprotection and oxidative stress (Fig. 3).

Direct interaction of NO with mitochondrial proteins is not necessary for NO to elicit cardioprotective effects on the mitochondrial redox environment. For example, the activation of NOS enzymes leads to the upregulation of both MnSOD and thioredoxin in a cGMP-dependent manner and is important in the second window of preconditioning (129). Lipid molecules are also affected by the redox environment of the mitochondrion. Although this is a relatively new field, the potential cardioprotective effects of nitro-linoleate (Fig. 2B) include cGMP-dependent vasodilatation (159), ligand activity for peroxisome proliferator–activated receptors (*e.g.*, PPAR-γ) (208), and induction of heme oxygenase (HO-1) (250). Interestingly, PPAR-γ ligands can reduce myocardial infarct size (246), and HO-1 has been implicated in cardioprotection during the second window of IPC (161).

Heat-shock proteins (Hsps) were initially characterized as inducible by heat stress, but now they are recognized to be expressed during hypoxia, IR, inflammation, heavy metal toxicity, endotoxins, and ROS exposure (215). Mitochondria have constitutively active isoforms, including Hsp10, Hsp60, and GRP75, which are used to prevent protein aggregation inside the mitochondrial matrix. Upregulation of Hsp 32, 60, and 72 during IR was found to be cardioprotective in tissue that underwent mild hyperthermic stress before ischemia. Under these conditions, mitochondrial ultrastructure and mitochondrial respiratory complex activity were preserved when compared with tissue that did not undergo hyperthermic stress (203). A possible mechanism may include the ability of Hsp60 to increase eNOS activity and downregulate iNOS activity (47). Besides alterations in gene regulation, posttranslational modifications may also regulate heat-shock protein activity and would probably be more relevant with the acute window of preconditioning. Proteomic studies have identified Hsp60 and GRP75 as being S-nitrosated, which may suggest a role for NO-dependent regulation of Hsps (104).

Krebs cycle

The Krebs cycle comprises a series of enzymes that are rich in both metal centers and reactive thiols, making them prime targets for regulation by RNS. However, direct interaction of the Krebs cycle with RNS is usually reported as a marker for oxidative stress, as seen with the nitration and irreversible inhibition of pyruvate dehydrogenase (166) and aconitase (101) (Fig. 3). Certain Krebs cycle enzymes have previously been shown to be reversibly regulated by ROS, including aconitase, α-ketoglutarate dehydrogenase, and succinate dehydrogenase (6, 41, 182). Besides limited information regarding the interactions of HNO with complex II (succinate dehydrogenase) (212), little evidence exists that RNS can affect other Krebs cycle enzymes. However, it is possible that reversible inhibition of the Krebs cycle would result in the slow reintroduction of electrons into the ETC and the gradual wakeup of mitochondrial metabolism on reperfusion.

Mitochondrial K^+_{ATP} channel

One of the most important mitochondrial proteins recognized to be involved in preconditioning is the K^+_{ATP} channel. The actual mechanism by which K^+_{ATP} channel opening elicits cardioprotection is not yet clear, and debate surrounds the order of events with respect to ROS generation (*i.e*., evidence suggests that ROS generation is an upstream trigger for K^+_{ATP} channel opening, and that K^+_{ATP} channel opening is a trigger for ROS generation) (78). Nevertheless, from the standpoint of NO signaling, several studies showed that NO can directly affect the K^+_{ATP} channel *via* S-nitrosation (Fig. 3) (205). In addition, the mito-K^+_{ATP} channel is thought to be a downstream target for PKG, in classic cGMP/NO signaling (60). However, in the latter case, the mechanism by which PKG signaling is transmitted into the mitochondrion remains unclear (144). Furthermore, all studies on the K^+_{ATP} channel are subject to several caveats. First, much controversy surrounds the actual existence of bona fide K^+_{ATP} channel subunits (KIR, SUR) in mitochondria, with conflicting results surrounding the use of antibodies and Western blots (5, 151). Second, many of the pharmacologic tools used to probe K^+_{ATP} channel function are nonspecific. Most notably, the K^+_{ATP} channel opener diazoxide is a known inhibitor of complex II (206), and a protonophoric uncoupler (121), whereas the K^+_{ATP} channel blocker 5-HD is a β-oxidation substrate (102). The non–K^+_{ATP} channel effects of both diazoxide and 5-HD were recently reviewed extensively (75, 102, 103), with the result that a large proportion of the literature on this topic may require reassessment. The mechanism of K^+_{ATP}-mediated protection is thought to include mild mitochondrial swelling, which may affect the formation of the PT pore (24). Although it has been argued that small K^+ fluxes that would result from opening of K^+_{ATP} channels (and K^+_{Ca} channels) in IPC would mildly uncouple mitochondria, it should be recognized that the magnitude of such K^+ fluxes is not large enough to account for the magnitude of H^+ leak seen in IPC (175).

Ca^{2+} handling

It is becoming increasingly obvious that the entire Ca^{2+}-handling machinery of the cell is sensitive to redox regulation, and it appears that mitochondria are no different in this regard. Proteins relevant to Ca^{2+} handling that are regulated by NO include the L-type Ca^{2+} channel, TRP channels, RyR, and K^+_{ATP} channels (205, 222, 224, 257). It was recently reported that mitochondria also contain an RyR (19), although it is not known if this is NO sensitive.

From a purely bioenergetic standpoint, the simple observation that both mitochondrial Ca^{2+} uptake and efflux depend on $\Delta\Psi_m$ is enough to explain most of the effects of NO on mitochondrial Ca^{2+} (*i.e.,* NO inhibits respiration, which de-energizes the mitochondrial membrane, thereby limiting Ca^{2+} uptake) (33). However, a number of other $\Delta\Psi_m$-independent mechanisms exist by which NO could affect mitochondrial Ca^{2+} uptake, including the possible S-nitrosation of mitochondrial Ca^{2+}-uptake proteins (Fig. 3). The molecular identities of the mitochondrial Ca^{2+}-uptake mechanisms (mitochondrial uniporter, rapid-mode uptake (RAM), and the mitochondrial ryanodine receptor) remain to be defined, but like other Ca^{2+} channels (224), could be targets of S-nitrosation.

PT pore formation and apoptosis

The overproduction of ROS and mitochondrial Ca^{2+} overload are known to trigger PT pore formation. Downstream effects as a result of PT pore opening include cytochrome *c* release, mitochondrial swelling, membrane depolarization, inhibition of ATP production, caspase activation, apoptosis and/or necrosis, and additional ROS production, which initiates mitochondrial and cellular dysfunction.

At pathologically high levels, $NO^{\cdot}$ can induce apoptosis both *via* the activation of p53, and *via* release of cytochrome *c* from the mitochondrial inner membrane and activation of caspases (145, 194, 253). Although high levels of $NO^{\cdot}$ can cause PT pore formation, smaller concentrations of $NO^{\cdot}$ inhibit the PT pore (33). Lower levels of $NO^{\cdot}$ protect the mitochondrion against apoptotic agents such as TNF-α, serum starvation, hypoxia, and H_2O_2 (97, 194). Mechanisms by which NO inhibits PT pore formation include the prevention of Bcl-2 cleavage by caspase 3 (145), mild dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$), and inhibition of Ca^{2+} uptake (33). In terms of cardioprotection, the $NO^{\cdot}$ donor DETA-NONOate was found to inhibit cyclosporin A–sensitive Ca^{2+}-induced mitochondrial swelling in mitochondria isolated from hearts subjected to IR (33, 243). Another study observed that aged endothelium, with decreased eNOS activity, and eNOS knockout mice were more susceptible to proapoptotic stimuli, which were reversed by NO donors (118).

Understanding how NO regulates the cell-death cascades and PT pore formation is essential in understanding NO-dependent cardioprotection. For example, caspases 3 and 9 are inhibited by S-nitrosation (164) and are probably the best-described mechanisms by which NO elicits an antiapoptotic signal. The direct effects of NO on PT pore regulation are as questionable as the components of the PT pore itself, but cannot be disregarded. The molecular composition of the PT pore remains a subject of considerable debate and is thought to include such components as the voltage-dependent anion channel (VDAC) of the outer membrane, the ANT of the inner membrane, and cyclophilin D of the mitochondrial matrix (98, 99). Recent attention has focused on mitochondrial "contact sites" (areas

where the inner and outer mitochondrial membranes are in close proximity), as regulators of PT pore formation (24). Because VDAC and ANT reside in the outer and inner membrane, respectively, it is thought that they regulate the PT pore through contact-site formation. It should be noted that recent studies using VDAC and ANT knockout mice have called into question the role that these proteins play in PT pore formation (11, 147), with the current consensus being that, in these knockout animals, other mitochondrial proteins may be able to substitute for the roles of ANT and VDAC, to reconstitute an active PT pore. Nevertheless, the ANT is known to contain PT pore-regulatory thiols that are redox sensitive (61). Currently, it is not known if the ANT thiols are direct targets for RNS (Fig. 3), because the extreme hydrophobicity of ANT renders it refractory to the proteomic tools typically used to identify NO modifications (*e.g.*, 2D gels). In addition to the ANT, creatine kinase (CK) also is associated with mitochondrial contact-site formation and is targeted for modification by NO (93) and $ONOO^-$ (217). Thus, the possibility of NO directly inhibiting PT pore opening by regulating CK and contact-site formation is also raised (Fig. 3).

H^+ leak

Mild proton leak (uncoupling) is a cardioprotective phenomenon found in IPC. H^+ leak is the permeability of the mitochondrial inner membrane to protons and is regulated by several proteins, including the ANT and the uncoupling proteins (UCPs) (77). UCPs were originally discovered in brown adipose tissue (UCP-1), but in 1997, several laboratories (84, 153) reported the existence of UCP homologues in other tissues, including the heart. UCPs contain several well-conserved solvent-accessible Cys residues, and UCP3 contains a PKG consensus phosphorylation sequence. In addition, several stimuli that upregulate NOS also increase UCP expression, and $NO^\cdot$ has been shown to downregulate UCP2 expression (32).

It is known that ROS and electrophilic oxidized lipids (*e.g.,* 4-HNE) can activate UCPs (23). However, the recent discovery of nitro-lipids, which (like 4-HNE) are electrophilic, raises the possibility that NO-derived reactive lipid species may activate UCPs. Notably, $ONOO^-$ is known to stimulate mitochondrial H^+ leak (31), and although this oxidant is traditionally regarded as detrimental to mitochondrial function, studies suggest that $ONOO^-$ may be cardioprotective under certain conditions (33, 154, 179, 180). Thus, a small H^+ leak may account for some of these protective effects of RNS.

Several strategies that upregulate H^+ leak have been shown to be cardioprotective, including uncoupling reagents such as FCCP and DNP (25, 26, 169) and transgenic overexpression of UCPs (116, 228). The cardioprotective effects of a mild H^+ would include the attenuation of ROS and mitochondrial Ca^{2+} by a small dissipation of $\Delta\Psi_m$ (27, 258). This is seen after IPC and has been associated with the activation of UCP2. The buildup of ischemic metabolites such as AMP can initiate IR-induced H^+ leak *via* activation of ANT (175). Whereas a mild H^+ leak is seen as cardioprotective, a large IR-induced H^+ leak is seen as deleterious, and appears to be related to PT pore formation (175).

Thus, overall, NO can act on several targets within the mitochondrion, and importantly, most if not all of these targets are implicated in IR injury and IPC. Therefore, the ability of NO to elicit cardioprotection would appear to be mediated at least in part by its direct effects on the mitochondrion. Having established the mitochondrion as a target for the cardioprotective effects of NO, we now turn our attention to the delivery of NO as a therapeutic molecule.

$NO^\cdot$ DONORS

A number of NO donors have proven to be invaluable tools in studying the effects of NO on the mitochondrion (74, 76, 83, 138, 198, 242). In addition, NO donors have been developed as cardioprotective agents. This section reviews some commonly used NO donors that afford cardioprotection in IR injury, and although a number of these compounds have been reviewed before, the information provided focuses on how the metabolism of each compound will affect mitochondria.

Organic nitrates and nitrites

Commonly used nitrates and nitrites include nitroglycerin, amyl nitrite, and isosorbide mono- and dinitrates. Nitroglycerin has been used to treat angina patients since 1857 (18, 177), and its metabolism is a complex process, which involves bioactivation (172) by mitochondrial aldehyde dehydrogenase isoform 2 (ALDH-2). Interestingly, the liberation of $NO^\cdot$ during metabolism is not necessary to provide protection, suggesting the formation of another cardioprotective NO derivative, such as NO_2^- (50). Nitrate tolerance is the main drawback to organic nitrate therapy, and although far from being completely understood, the mechanism of tolerance appears to involve the inhibition of mitochondrial ALDH-2 by the generation of ROS, $ONOO^-$, and even nitroglycerin itself (172).

Nitrosyl complexes

The production of $NO^\cdot$ by sodium nitroprusside (SNP) is dependent on the $NO^\cdot$ release from a square bipyramidal ferrous iron and cyanide complex, *via* a one-electron transfer by a reducing agent, or on exposure to light. Actually, SNP is a source of NO^+, which is a nitrosating species and can posttranslationally modify amino acids (2). The primary downfall of SNP is CN^- release on the release of $NO^\cdot$. The dosage for SNP is usually small enough for the released CN^- to be metabolized to thiocyanate by a mitochondrial enzyme in the liver (rhodanase). However, if problems exist with this metabolic pathway, SNP treatment can lead to CN^- poisoning *via* inhibition of mitochondrial cytochrome *c* oxidase (complex IV). Evidence indicates also that SNP may be involved in mutagenesis (177).

S-nitrosothiols (RSNOs)

Common cardioprotective SNOs include *S*-nitrosoglutathione (GSNO), *S*-nitrosopenicillamine (SNAP), *S*-nitrosohemoglobin (SNO-Hb), *S*-nitrosocysteine, and SNO-albumin. The administration of RSNOs before ischemia has been found to be cardioprotective in a number of models of IR injury (16, 148, 174). The transport and metabolism of SNOs in the cardiomyocyte appears to be dependent on the individual SNO. For ex-

ample, the transport of Cys-NO has been reported to involve the L-amino acid transporter (261). However, GSNO is thought to liberate $NO^{\cdot}$ outside the cell and generate nitrosating species (*e.g.*, N_2O_3) in cellular membranes (218). This adds to the complexity of NO-dependent signaling and gives rise to the possibility that different S-nitrosating agents probably control different targets both within and outside the cell.

One SNO of particular interest is SNO-Hb, because it has been proposed (although recently questioned) that this species may act as an O_2-sensitive NO donor within the circulation (216). It is hypothesized that the R → T state transition of Hb, which occurs on deoxygenation ($HbO_8 \rightarrow Hb$), favors the release of NO from the SNO at the β^{93} cysteine. Thus, NO is released only under hypoxic conditions, leading to the idea that SNO-Hb acts as an antihypoxic signal, by selectively delivering NO to hypoxic areas, causing vasodilatation that reverses the hypoxia. Several issues with this hypothesis include large variations in the amount of SNO-Hb reported *in vivo*, along with a lack of identity for the precise NO-related species that is formed on SNO-Hb breakdown (90). In addition, how NO actually exits the red blood cell is unclear, given that oxy-Hb itself is a sink for NO. Nevertheless, it was recently reported that administration of SNO-Hb can directly elicit cardioprotection from IR injury (8, 176).

Nitric oxide–releasing nonsteroidal antiinflammatory drugs (NO-NSAIDS)

The NO-NSAIDs belong to two categories, which are either SNO derivatives or nitrate ester derivatives. Originally, the NO-NSAIDs were developed to reduce the gastrointestinal toxicity of the NSAIDs (82). The generation of NO-NSAIDs resulted in a dual-action drug that, when metabolized, had antiinflammatory effects and generated $NO^{\cdot}$. The $NO^{\cdot}$ released from the NO-NSAIDs has been found to elevate both SNOs and NO_x in the plasma and the urine (82). In terms of $NO^{\cdot}$-dependent cardioprotection, NO-NSAIDs, such as 2-acetoxybenzoate 2-[1-nitroxy-methyl]-phenyl ester (NCX 4016), have been found to S-nitrosate and inhibit caspases (81). Although these compounds release $NO^{\cdot}$ in the plasma to initiate cardioprotective responses in the endothelium, the ability of this $NO^{\cdot}$ to directly act on the cardiomyocyte or cardiac mitochondria is not known.

1-substituted diazen-1-ium-1,2-diolates (NONOates)

The NONOates were developed during the 1990s (165) and can be divided into $NO^{\cdot}$ and HNO donors (170). These compounds are stable at alkaline pH, but break down at physiologic pH to release two equivalents of NO per molecule. The rate of decomposition is determined by the rest of the molecule, with half-lives varying from seconds (Proli-NONOate) to minutes (Spermine-NONOate), to hours (DETA-NONOate). The primary downfall of the NONOates is their release of $NO^{\cdot}$ in the bloodstream, and hence they initiate systemic vasodilatation. Angeli's salt, which is an HNO donor (NONOate), is discussed later.

Nitrite (NO_2^-)

Nitrite is a stable NO species found throughout the circulation at micromolar levels. However, in the presence of a nitrite reductase, such as deoxy-Hb, NO_2^- is converted to $NO^{\cdot}$, and therefore serves as a circulating $NO^{\cdot}$ pool (59). The use of NO_2^- as a cardioprotective agent has been seen in experiments in which intraperitoneal administration of NO_2^- to mice decreases the amount of necrosis and tissue damage seen after IR injury of the heart or liver (76). Furthermore, recent findings suggest that NO_2^- delivery in drinking water may elicit similar protective effects (211). The concept of NO_2^- as an $NO^{\cdot}$ donor has many of the same limitations as SNO-Hb, in that the NO (or redox equivalent thereof) still has to exit the erythrocyte to act as a signal. However, other proteins within the cellular milieu also can serve as nitrite reductases, such as myoglobin (213) and complex IV (46). The fact that complex IV can both make and be inhibited by $NO^{\cdot}$ serves to highlight NO_2^- as another NO donor, the reactivities of which are associated with and tightly controlled by the mitochondrion.

Peroxynitrite ($ONOO^-$)

Like other NO-related molecules, peroxynitrite ($ONOO^-$) is a double-edged sword in cardiovascular biology. The formation of $ONOO^-$ involves the rapid reaction of $NO^{\cdot}$ with superoxide ($O_2^{\cdot -}$) ($\sim 5 \times 10^9$ M/sec) (197), and compounds such as Sin-1, which produce both $O_2^{\cdot -}$ and $NO^{\cdot}$, have been used as $ONOO^-$ donors to study $ONOO^-$-mediated events. The mitochondrion, being a source of ROS, is both a site of $ONOO^-$ generation, and a target for $ONOO^-$ modification. At the mitochondrial level, $ONOO^-$ can cause such events as complex I S-nitrosation (37), PT pore formation (29), nitration of Mn-SOD (168), and activation of H^+ leak (31). In contrast, $ONOO^-$ has been reported to be cardioprotective when administered in small concentrations (154, 179, 180, 197). However, downfalls of studying $ONOO^-$ in biologic systems include delineating the effects of $ONOO^-$ from its decomposition products (*e.g.*, $OH^{\cdot}$ and $NO_2^{\cdot}$) and the probability of direct $ONOO^-$ modification *in vivo* (170) [nitration of MnSOD (168)]. Furthermore, it should be emphasized that although $ONOO^-$ can be chemically synthesized and administered *in vitro*, $ONOO^-$ *in vivo* cannot exist without the prior existence of $NO^{\cdot}$, and the fact that these two molecules ($NO^{\cdot}$ and $ONOO^-$) react with each other, makes the translation of *in vitro* experiments with pure $ONOO^-$ very difficult (152).

Nitroxyl (NO^-)

Nitroxyl is generated from the one-electron reduction of $NO^{\cdot}$ and has recently emerged as an important redox congener of $NO^{\cdot}$. The pK_a of NO^- has been reported to be 11 (212), and therefore, under physiologic conditions, NO^- is protonated (HNO). To study the biologic effects of HNO, donors such as Angeli's salt and Piloty's acid have been used. In terms of IR injury, HNO has been reported to be the counterbalance of other $NO^{\cdot}$ donors, such as Deta-NONOate, by contributing to deleterious biomolecular oxidation (249). However, recent studies have found HNO to be a cardioprotective agent when administered under suitable conditions (186). This is seen with the administration of Angeli's salt (1 μM) before IR, which has been found to improve contractility and limit infarct size (184). Regulation of complex II as described earlier may prove to be one HNO-dependent cardioprotective mechanism (212).

Endogenous NO signaling

As highlighted in Fig. 1, several endogenous signaling reactions can lead to the upregulation of eNOS, or enhancement of $NO^{\cdot}$ bioavailability, which is cardioprotective. Administration of antioxidants, statins, ACE inhibitors, angiotension II–receptor blockers, Ca^{2+} channel blockers, and β-blockers are examples of indirect $NO^{\cdot}$ donors that cause an upregulation of eNOS (201).

DELIVERING $NO^{\cdot}$ TO MITOCHONDRIA

For a number of reasons, delivering NO to the mitochondrion would be cardioprotective. First, targeting the mitochondria may avoid potentially deleterious side effects of delivering $NO^{\cdot}$ systemically, such as vasodilatation, which would affect cardiac afterload and/or preload and affect contractile function. Second, one could decrease the dosage, as is the case with any targeted drug. Last, targeting molecules such as RSNOs to the mitochondrion would serve as a two-for-one therapeutic, because metabolism of RSNOs would result in a NO-dependent signal along with the regeneration of a parent thiol that can serve as an antioxidant. The supplementary antioxidant may serve to help maintain the redox status and reverse NO-dependent modifications on reperfusion. Several antioxidant molecules have been targeted to the mitochondria (62, 142, 262) and are currently providing starting points for the development of mitochondrially targeted NO donors. Strategies for delivering NO to the mitochondria include using molecules that accumulate in mitochondria because of their hydrophobicity and positive charge, molecules that enter mitochondria *via* carrier proteins unique to the organelle, and prodrugs that are metabolized by uniquely mitochondrial enzymes to reveal an active entity.

With these strategies, several mitochondrially targeted NO donors have been patented (US-20060122267) and are being synthesized (Fig. 5). The best characterized of this series of compounds is *S*-nitroso-mercaptopropionyl glycine (SNO-MPG). The parent compound (mercaptopropionyl glycine, MPG) was used to develop a mitochondrial S-nitrosating agent based on its structural similarities with mitochondrial substrates (51). Administration of SNO-MPG to isolated mitochondria, cardiomyocytes, and Langendorff-perfused hearts was found to enhance mitochondrial S-nitrosation, complex I inhibition, and cardioprotection, when compared with S-nitrosating agents that did not preferentially accumulate inside mitochondria (174). The use of MPG itself has been found to be cardioprotective in IR injury (227, 232), but administration of equivalent or lower concentrations of SNO-MPG before ischemia enhanced cardioprotection. The development of drugs such as SNO-MPG may provide a new paradigm in NO-dependent cardioprotection.

It should be noted that obstacles remain to be faced on the road to further developing mitochondrially targeted NO donors. This includes how the molecule will reach the mitochondrion *in vivo*. Among the NO donors described earlier, many of the downfalls arise from the lack of ability of the molecule to leave the plasma and endothelium to reach cardiomyocyte mitochondria. Developing NO donors that generate more-stable intermediates, such as SNOs or nitrite, may therefore be more practical than developing donors that generate $ONOO^{-}$ and

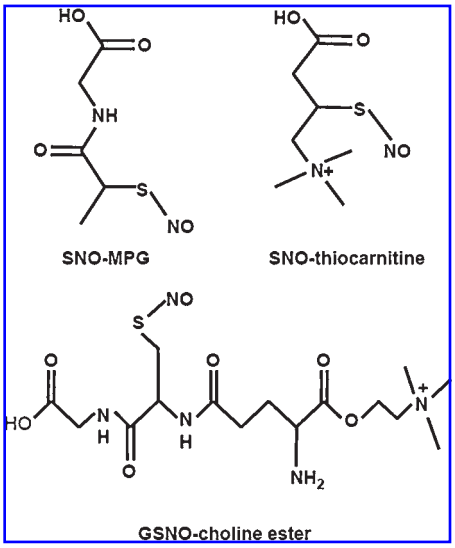

FIG. 5. Mitochondrially targeted $NO^{\cdot}$ donors. Mitochondrial NO donors have been developed (U.S. patent 20060122267) and are designed to target the mitochondria based on membrane potential, hydrophobicity, and substrate specificity. The cardioprotective effects of SNO-MPG (174) have been the best characterized, leading to enhanced mitochondrial S-nitrosation and cardioprotection when compared with treatments using the parent thiol (MPG) or GSNO. *S*-nitroso-thiocarnitine and GSNO-choline ester are currently under investigation to see if they elicit a similar response.

$NO^{\cdot}$ within the vascular space. The ability of these compounds to cross the blood–brain barrier may raise further complications, because it was shown that complex I is reversibly inhibited after administration of SNO-MPG (174), and it is known that complex I inhibitors such as rotenone can lead to Parkinson disease (120). If SNO-MPG is administered on a daily regimen to patients, similar to nitroglycerin, and SNO-MPG can readily cross the blood–brain barrier, the possibility exists that patients could potentially have complex I deficiencies such as Parkinson disease. This applies not only to NO and SNO, but also to all ETC-inhibition–based therapies. Another obstacle involved with any NO-based therapy is the issue of tolerance, most obviously seen with nitroglycerin (see earlier).

NO EFFECTS ON PERIPHERAL AND TEMPORALLY REMOVED RESPONSES TO IR INJURY

Although much of the discussion in this review focused on the acute effects of NO, and events at the mitochondrial level that occur during IR injury itself, it should not be forgotten that the response of the heart and the whole organism to IR injury is a prolonged process, which involves complex inflammatory responses, tissue remodeling, and neurohormonal changes (130, 134, 223). Nitric oxide intermediates have the potential to affect a number of these processes. For example, iNOS-derived $NO^{\cdot}$ has been reported to contribute to the development of congestive heart failure, which often occurs long after a myocardial infarction (69). Postischemic remodeling often includes

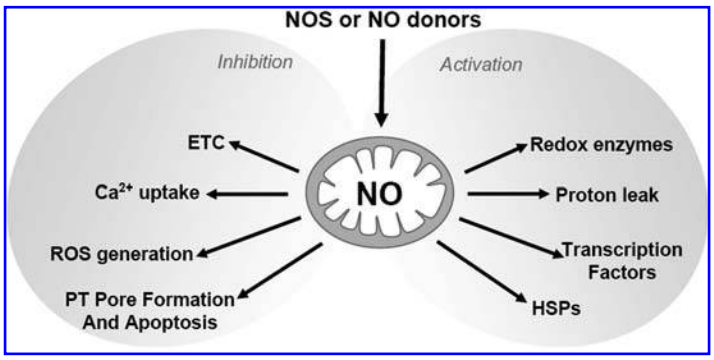

FIG. 6. Overview of NO-dependent mitochondrial cardioprotection. This figure serves to highlight that the cardioprotective effects of NO are mediated *via* a mix of both inhibitory and stimulatory actions on a variety of protein targets. Nitric oxide directly regulates mitochondrial functions, such as ETC activity, Ca^{2+} handling, and PT pore activation, which under the right conditions can protect the mitochondria from IR injury. The cardioprotective nature of NO is dependent on the ability of the species to modify a specific target and the ability of the mitochondrion to regulate the modification process.

cardiac hypertrophy, and we previously showed that an upregulation of iNOS leads to an NO-mediated bioenergetic defect in a rat model of hypertrophy (69). This is in agreement with studies suggesting that NO is an important factor governing mitochondrial function in human failing hearts (171). In contrast, overexpression or upregulation of iNOS has also been shown to be cardioprotective in models of heart failure or IR injury (114, 138). Mechanisms accounting for this cardioprotective effect may include the antiinflammatory effects of NO and the quelling of the adhesion of neutrophils to the reactive endothelial surface in the 24–48 h after an MI. Similarly, although systemic vasodilatation induced by NO may help the recovery of the heart by reducing its workload, too little venous return would also lead to improper ventricular filling, which could be detrimental to cardiac output. Thus, overall, careful timing of NO delivery before, during, and after IR injury is essential to gain the maximal therapeutic benefit.

CONCLUSIONS AND CLINICAL OUTLOOK

Nitric oxide and NO intermediates are essential in both pharmacologic (anesthetic) and ischemic preconditioning. Although the study of cardioprotective effects of NO has centered on the sGC signaling pathway, emerging evidence now shows that sGC-independent effects also are important. The mitochondrion is a cellular hub for both preconditioning and NO signaling (Figs. 1 and 6), and therefore represents a point of convergence in cardioprotection.

Currently, the clinical treatment of both acute myocardial infarction and elective cardiac ischemia (*e.g.*, during bypass surgery) draw on a very limited range of cardioprotective drugs. Mortality rates from cardiac ischemic injury remain high, despite several decades of research in this area. Recently, ischemic preconditioning and anesthetic preconditioning have begun to be applied in clinical settings (195). However, with this move from bench to bedside has come the realization that a variety of confounding factors may limit applicability of IPC and/or APC in humans. For example, it is known that cycloxygenase inhibitors can diminish the efficacy of APC (196). In addition, diabetics also appear refractory to APC and IPC (139, 141, 143). One interesting observation that emerges from Fig. 1 is that NO effects on mitochondria in IPC appear to bypass much of the cell-signaling "alphabet soup" that is essential for most preconditioning pathways. Thus, the possibility arises that if a given patient population is refractory to preconditioning, due to a defect in one of these signaling pathways (*e.g.*, defective insulin signaling in diabetics), then NO may be able to bypass such a defect and still elicit cardioprotection.

ABBREVIATIONS

AC, adenylate cyclase; AIF, apoptosis-inducible factor; Akt, protein kinase B; $ALDH_2$, aldehyde dehydrogenase isoform 2; ANT, adenine nucleotide translocase; APC, anesthetic preconditioning; Bcl, B-cell lymphoma family protein (Bcl-2, Bax, *etc.*); cGMP, cyclic guanosine monophosphate; CK, creatine kinase; CoQ, coenzyme Q; CsA, cyclosporin A, Cys-NO, *S*-nitrosocysteine; DAG, diacylglycerol; DNP, 2,4-dinitrophenol; eNOS, endothelial NOS; ETC, electron-transport chain; $FADH_2$, flavin adenine dinucleotide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; GF, growth factor; GFRs, growth factor receptors; Glrx1, glutaredoxin isoform 1; GPCR, G protein–coupled receptor; GRP75, glucose-regulated protein 75; GSH, glutathione; GSK-3β, glycogen synthase kinase 3β; GSNO, *S*-nitrosoglutathione; HSPs, heat-shock proteins; HSP70, heat-shock protein 70; H_2O_2, hydrogen peroxide; IPC, ischemic preconditioning; IP3, inositol triphosphate; IR injury, ischemia–reperfusion injury; K^+_{ATP}, ATP-sensitive K^+ channel; K^+_{Ca}, Ca^{2+}-sensitive K^+ channel; L-NAME, *N*(G)-nitro-L-arginine methyl ester; LNO_2, nitrated lipids; MAPK, mitogen-activated kinases (ERK1/2, p38, *etc.*); MCU, mitochondrial uniporter; MnSOD, manganese superoxide dismutase; MPO, myloperoxidase; mtNOS, mitochondrial NOS; mTOR, mammalian target of rapamycin; NADH, nicotinamide adenine dinucleotide; NCX 4016, 2-acetoxybenzoate 2-[1-nitroxymethyl]-phenyl ester; $NO^{\cdot}$, nitric oxide; NO, nitric oxide derivative; NO^+, nitrosonium cation; NO^-, nitroxyl anion; NO_2^-, nitrite; $NO_2^{\cdot}$, nitrous oxide; NONOates, 1-substituted diazen-1-ium-1,2-diolates; MI, myocardial infarction; NOS, nitric oxide synthase; NO-NSAIDs, nitric oxide–releasing nonsteroidal antiinflammatory drugs; N-Tyr, tyrosine nitration; $O_2^{\cdot -}$, superoxide radical; $OH^{\cdot}$, hydroxyl radical; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; Ox-Phos, oxidative phosphorylation; p70s6k, 70-kDa ribosomal protein S6 kinase; p90rsk, 90-kDa ribosomal S6 kinase (MAPKAPK1); PARP, poly(ADP-ribose) polymerase; PDE, phosphodiesterase; PDK-1, PI3K-dependent protein kinase 1; PGC-1α, peroxisome proliferator–activated receptor-γ coactivator; PI3K, phospho-

inositide-3 kinase; PJ-34, *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N*,*N*-dimethylacetamide.HCl; PKA, cAMP-dependent protein kinase; PKCδ/ε, Ca^{2+}- and diacylglycerol-dependent protein kinase (δ/ε isoforms); PKG, protein kinase G; PLC/PLD, phospholipase C/D, PPC, pharmacologic preconditioning; PT pore, permeability transition pore; PTEN, phosphatase and tensin homologue; RaM, rapid-mode uptake; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSNOs, *S*-nitrosothiols; RyR, ryanodine receptor; SfA, sanglifehrin A; sGC, soluble guanylate cyclase; SNAP, *S*-nitrosopenicillamine; SNO-Hb, *S*-nitrosohemaglobin; SNP, sodium nitroprusside; Src, tyrosine kinase product of c-src gene; TNF-α, tumor necrosis factor-α; TRP channels, transient receptor potential channels; UCP, uncoupling protein; VDAC, voltage-dependant anion channel; x-PC, preconditioning; $\Delta\Psi_m$, mitochondrial membrane potential; 3-NP, 3-nitroproprionic acid; 4-HNE, 4-hydroxynonenal; 5-HD, 5-hydroxydecanoate.

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