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Nitrite as a mediator of ischemic preconditioning and cytoprotection

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Abstract

Ischemia/reperfusion (IR) injury is a central component in the pathogenesis of several diseases and is a leading cause of morbidity and mortality in the western world. Subcellularly, mitochondrial dysfunction, characterized by depletion of ATP, calcium-induced opening of the mitochondrial permeability transition pore, and exacerbated reactive oxygen species (ROS) formation, plays an integral role in the progression of IR injury. Nitric oxide (NO) and more recently nitrite (NO₂⁻) are known to modulate mitochondrial function, mediate cytoprotection after IR and have been implicated in the signaling of the highly protective ischemic preconditioning (IPC) program. Here, we review what is known about the role of NO and nitrite in cytoprotection after IR and consider the putative role of nitrite in IPC. Focus is placed on the potential cytoprotective mechanisms involving NO and nitrite-dependent modulation of mitochondrial function.

Introduction

Ischemia/reperfusion (IR) injury underlies the progression of a number of pathologies in various organs and is a significant cause of morbidity and mortality. For example, each year over one million people suffer from acute myocardial infarctions in the United States, and beyond the heart, IR injury contributes to complications of stroke and solid organ transplant. Despite years of research, few pharmacological agents that successfully prevent IR injury have been identified. Interestingly, the cardioprotective effects of nitric oxide (NO) and its metabolite nitrite (NO₂⁻) have been recognized for centuries. For example, the use of sublingual saltpeter (potassium nitrite) for the treatment of angina in China dates back to the eighth century [1] while nitroglycerin has been used since the early 1800s [2]. However, the basic mechanisms by which NO and nitrite mediate their protective effects are still being elucidated. Moreover, the role these molecules play in physiological adaptation to ischemic stress remains unclear.

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Mitochondrial dysfunction is known to contribute to the progression of IR injury and a number of cytoprotective agents, including the highly protective ischemic preconditioning (IPC) program, mediate their action through mechanisms that converge at the level of the mitochondrion. NO is a well-characterized modulator of mitochondrial function and has been implicated in the signaling of IPC [3-4]. In contrast, nitrite has only recently been identified as an endocrine reservoir of NO that mediates physiological responses [5]. While physiological levels of nitrite have recently been shown to mediate cytoprotection after IR and the effects of nitrite on the mitochondrion are beginning to be elucidated, its involvement as an active signaling mediator in the IPC pathway has not been considered. In the following sections, we will review what is known about nitrite mediated cytoprotection after IR, and the putative mechanisms by which NO and nitrite mediate their actions, particularly through the modification of mitochondrial function. This article will focus on the established role of NO in IPC as well as the potential involvement of nitrite in this adaptive mechanism.

Ischemia/Reperfusion Injury and mitochondrial dysfunction

On a cellular level, several processes contribute to the progression of IR injury. Within the endothelium, IR is associated with the biosynthesis of adhesion molecules that mediate leukocyte adhesion and migration to the ischemic zone, which initiates and propagates tissue injury [6]. Oxidation of protein and lipids and a dysregulated inflammatory response eventually progress to cellular apoptosis and/or necrosis. On a sub-cellular level mitochondria are particularly susceptible to ischemia and contribute to the progression of IR injury. Under normoxic conditions, about 90% of metabolism in the heart and liver is aerobic, and fatty acids supply the major part of the energy requirements [7]. During ischemia, the shift from aerobic to anaerobic metabolism is energetically inefficient as oxidation of one mole of glucose to lactate yields only 7% of the ATP that is synthesized under aerobic conditions. Furthermore, products formed during anaerobic glycolysis, including lactic acid, protons and NADH, decrease intracellular pH [8-9]. Persistent ischemia or complete lack of oxygen and nutrients leads to discontinued ATP production, the consumption of high phosphate energy reserves, and ultimately the termination of ATP-dependent cellular functions.

During IR mitochondria are both a source of oxygen radicals and a target of ROS-induced damage. Under basal conditions, it is estimated that 1-2 % of the electron flow through the electron transport chain contributes to the formation of superoxide ($O_2^{\bullet-}$) [10-11]. Superoxide is readily removed by an efficient antioxidant network that includes manganese superoxide dismutases (MnSOD), glutathione peroxidase and peroxideroxins [12-13]. However, at the time of reperfusion, excess ROS production overwhelms cellular antioxidant defenses, leading to protein damage within and outside the mitochondrion. It is now recognized that increased ROS production during reperfusion is one of the major mechanisms underlying IR injury. The formation of oxygen-derived species has been detected at less than 10 seconds into reperfusion [14]. Paradoxically, there is also evidence for the formation of ROS during ischemia, even though minimal oxygen is present [15-17]. This is due to the accumulation of electrons along the respiratory chain as oxygen concentration drops. The reduced state of the mitochondrial complexes promotes the acceptance of electrons by the low concentrations of oxygen present, resulting in $O_2^{\bullet-}$ formation [18-19]. The main sites of ROS generation are mitochondrial complexes I and III, and several studies suggest that a temporal progression of $O_2^{\bullet-}$ formation exists during ischemia, which begins at complex I and later continues at complex III [20-21].

The mitochondrion is also a major instigator of post-ischemic cell death through not only increased ROS production, but also via opening of the permeability transition pore (mPTP)

and subsequent release of pro-apoptotic proteins such as cytochrome *c* in response to Ca_2^{2+} overload [22-24]. The influx of Ca_2^{2+} into the mitochondrion at reperfusion activates Ca_2^{2+} /Calmodulin-dependent proteolytic enzymes, and triggers the opening of the mitochondrial permeability transition pore (mPTP) [25-26]. The opening of the mPTP increases mitochondrial swelling as solutes equilibrate between the mitochondrial matrix and the cytoplasm. This swelling results in the disruption of the outer mitochondrial membrane and the exit of pro-apoptotic factors into the cytosol, including cytochrome *c* and apoptosis-inducing factor (AIF) initiating the mechanism of ATP-dependent apoptosis [25,27-28]. In the absence of ATP, cell death occurs via necrosis. In addition, adenine produced by ATP catabolism is transformed into hypoxanthine, which promotes xanthine oxidoreductase catalyzed ROS generation, further contributing to reperfusion injury.

Ischemic preconditioning and NO

One of the most potent cytoprotective strategies for IR identified to date is IPC, whereby several brief intervals of sub-lethal ischemia followed by reperfusion confer protection against a subsequent more prolonged ischemic insult. This phenomenon, first described by Murry and colleagues in dogs [29] has now been observed in several species, including humans [30-33]. Two temporal windows of protection have been described for IPC: an early or acute phase that lasts for 1–3 h following the IPC stimulus and a delayed phase of protection that begins 18–24 h after the initial IPC stimulus and persists for 24–72 h [34-35]. While the two windows of protection most likely share common signaling pathways and have both been shown to involve protection of mitochondrial function, the early/acute phase is thought to primarily involve posttranslational modification, whereas delayed preconditioning predominantly involves changes in gene expression and upregulation of cardioprotective proteins [36]. In the last 2 decades since the discovery of IPC, research has focused on uncovering the mechanism of IPC-mediated protection, with the purposes of understanding the physiological process of adaptation to ischemia as well as developing therapeutics mimicking the protective effects of IPC. While the exact signaling of this phenomenon remains unclear, several pharmacological mimetics and biological triggers of delayed IPC, including adenosine, opioids, bradykinin, cytokines, NO and ROS (recently reviewed in [35]), have been identified. These agents activate a number of upstream signaling cascades, several of which converge at the point of Nitric Oxide Synthase (NOS) activation, suggesting that NO production is a central event in delayed IPC. “The NO hypothesis of late preconditioning”, pioneered by Bolli and colleagues, suggests that NO plays a role both in initiating and mediating delayed cardioprotection [4]. According to this paradigm the IPC pathway is initiated by increased enzymatic NO synthesis by endothelial NOS (eNOS) which acts as a trigger for downstream signaling that culminates in the upregulation of cardioprotective mediators, particularly the inducible form of NOS (iNOS) [4]. While the mechanisms by which eNOS-derived NO triggers the delayed window of protection are still being elucidated, several pathways have been implicated. A number of studies now show that the early production of ROS is integral in signaling leading to delayed cytoprotection. Phosphorylation of protein kinase C, recruitment of tyrosine kinases, and activation of NF- κ B also play a role. Activation of these early pathways ultimately regulates gene expression of cardioprotective proteins [36]. It has also recently been proposed that stimulation of the mitochondrial biogenesis, resulting in remodeling of the structure and/or function of the mitochondrion, may be one of the downstream consequences of IPC [37]. Studies demonstrating that NO is a potent stimulator of mitochondrial biogenesis are consistent with the NO hypothesis of IPC [38]. While it is unknown exactly how upregulation of iNOS mediates cytoprotection or why this complex mechanism of eNOS stimulated iNOS upregulation is necessary, data accumulated over the last decade clearly support the concept that the late upregulation of iNOS is integral to the protective effects of delayed IPC [4,36].

While the role of eNOS and iNOS in IPC are relatively well characterized, the role of neuronal NOS (nNOS) in IPC is not as clear. The role for nNOS in IR is controversial as early studies showed that mice lacking nNOS sustained more cerebral ischemic injury[39], while the same strain of mice showed no change in their response to myocardial ischemia[40-41]. Additionally, other studies demonstrated that nNOS mRNA and protein were upregulated at the end of the late phase of IPC (72 hours) and that this upregulation was important in IPC-mediated protection of the heart[42]. More recent studies have shown that while inhibition of nNOS is protective in a model of myocardial ischemia, its activity is required to observe the protective effects of delayed IPC[43-44]. While the mechanism by which nNOS mediates cytoprotection during IPC is not yet clear and are currently being investigated, these studies demonstrate that the activation of the MAP kinase p38 is involved in the protective effect[43].

A role for nitrite in delayed ischemic preconditioning

The role of NO in IPC was uncovered primarily through the measurement of the levels and enzymatic activities of eNOS and iNOS as well as manipulation of these enzymes by knockout or transgenic overexpression strategies in animal models of IPC-mediated protection. Seminal studies have shown that iNOS mRNA and protein expression increase after administration of IPC, resulting in increased iNOS activity and tissue nitrite/nitrate levels. Additionally, the cardioprotective effects of delayed IPC were lost in a murine model of iNOS deficiency or after treatment of wild type animals with an iNOS inhibitor [45-46]. In conjunction with changes in NOS activity, the role of NO in delayed IPC was solidified by the observation that nitrite and nitrate levels were increased after administration of IPC [46]. While these end products of NO oxidation were assessed as stable surrogates for NO formation, accumulating evidence in the last decade demonstrates that these species are not physiologically inert as once believed, particularly in hypoxic or ischemic conditions. Nitrite is now accepted as an endocrine reservoir of NO that can be reduced to bioavailable NO as oxygen concentrations and pH decrease over a physiological gradient [5]. A number of proteins, including deoxygenated hemoglobin and myoglobin, cytochrome *c*, cytochrome *c* oxidase, eNOS, carbonic anhydrase and xanthine oxidoreductase, have been identified as nitrite reductases, which catalyze the reduction of nitrite in the blood and tissues [5,47-50]. Once bioactivated, nitrite mediates physiological responses such as hypoxic vasodilation [51], stimulation of angiogenesis [52], modulation of mitochondrial respiration [49,53] and increases in exercise tolerance [54]. Moreover, in the last six years, several studies from multiple laboratories have demonstrated that low concentrations of nitrite mediate potent cytoprotection in a number of organs and animal models of IR (summarized in Table 1).

The finding that nitrite modulates resistance to ischemic stress in conjunction with previous work showing that IPC leads to the augmentation of both iNOS expression and nitrite levels has led many investigators to hypothesize that nitrite may be an integral mediator of delayed IPC. In this scenario, the upregulation of iNOS may serve as a mechanism to generate sufficient quantities of nitrite for cytoprotection. Indeed, previous studies have shown that after the administration of IPC, the total tissue concentration of nitrite and nitrate in the heart increases by approximately 2nmol/mg [55]. Based on our prior studies suggesting that a change in cardiac nitrite concentration of less than 20 pmol/mg protein mediates cytoprotection [56], the concentrations of nitrite generated during IPC appear to be in the protective range. Consideration of nitrite as an active cytoprotective mediator presents a clear advantage over NO in the context of ischemia, as nitrite remains a stable storage form of NO that is selectively bioactivated in conditions of hypoxia and low pH (both conditions are present in ischemia). Thus, nitrite may serve as an “ischemic NO buffer” that accumulates for hours to days after IPC is administered but only exerts its action once the ischemic insult is initiated. The stability of nitrite in normoxia may contribute to the long

temporal window (42-72 hours) in which delayed IPC is cytoprotective. In contrast to the stimulation of nitrite-dependent NO formation during hypoxia, NOS dependent NO formation is inhibited during ischemia. This is due to the fact that oxygen is a requisite substrate for enzymatic NO generation, and while the K_M of oxygen for iNOS is controversial, it is estimated to be approximately 25 μM [57], well above the concentration of oxygen present during ischemia.

In the following sections we describe the pathways by which nitrite is generated and bioactivated in blood and tissues, and describe what is known about nitrite-mediated protection after IR. We then consider the putative cellular targets for NO and nitrite within the mitochondrion whose modulation potentially leads to resistance from ischemic stress.

Nitrite formation and reduction

Formation of endogenous nitrite

Endogenous nitrite levels range from 100nM in the blood to up to 25 μM in the heart and liver and derive from both the oxidation of NO as well as dietary sources [58-59]. Approximately half of basal nitrite originates from the auto-oxidation of NOS-dependent NO. However, the third order reaction of NO with oxygen ($k = 10^6 \text{ M}^{-2}\text{s}^{-1}$) is kinetically unfavorable to the reaction of NO with heme proteins such as hemoglobin in blood or cardiac myoglobin ($k = 10^7 \text{ M}^{-1}\text{s}^{-1}$) which yield nitrate. Thus, it appears that catalysis of the auto-oxidation reaction is required in order to generate significant concentrations of blood and tissue nitrite. Consistent with this hypothesis, it has recently been shown that the multicopper oxidase, ceruloplasmin, catalyzes the reaction of NO with oxygen in the plasma. The active site of the enzyme, which consists of a copper tricluster, coordinates NO and catalyzes the one electron oxidation of NO to NO^+ , which is then rapidly hydrated to nitrite in the plasma [60]. Ceruloplasmin is endogenously present at 1-3 μM in the plasma. However, the protein can be rapidly upregulated in conditions of inflammation as well as after ischemia to levels of 20 μM [61]. It is interesting to speculate that as an acute phase protein, ceruloplasmin expression may be upregulated after the administration of IPC resulting in an increase in the fraction of NOS-derived NO that is oxidized to nitrite. This may serve as a physiological adaptive mechanism after acute ischemia, in which nitrite is accumulated to mediate protection against a subsequent ischemic insult.

The remaining half of endogenous nitrite derives from dietary sources of nitrite and nitrate including cured meats and leafy green vegetables. Dietary nitrate is converted to nitrite by a complex gastroenteric cycle in which symbiotic bacterial flora which contain nitrite reductase enzymes reduce nitrate to nitrite. The salivary nitrite and nitrate is swallowed and the unreduced nitrate rapidly absorbed by the gut into the bloodstream. This nitrate is then concentrated by the salivary gland and secreted into the saliva at a concentration of 2-8mM. This cycle is reviewed more extensively in [5].

Consistent with the cytoprotective function of nitrite, modulation of basal nitrite concentrations through the alteration of either dietary nitrite/nitrate or ceruloplasmin expression alters the response to ischemic stress [56,58,60]. We have previously shown that ceruloplasmin knockout mice, which have decreased plasma nitrite concentrations, sustain more injury after hepatic IR compared to wildtype mice. Administration of nitrite by intraperitoneal injection to the ceruloplasmin deficient mice during hepatic IR attenuated injury to the level of wildtype mice undergoing identical IR surgery, suggesting that the exacerbated injury was due to depleted basal nitrite levels [60]. Similarly, studies have now demonstrated in murine models of hepatic and myocardial IR that depletion of dietary nitrate/nitrite results in exacerbation of IR injury, while dietary supplementation attenuates injury in these models [56,58]. Most recently, in a clinical study subjects whose endogenous

nitrite levels were increased after drinking beetroot juice (which contains high concentrations of nitrate) showed resistance to acute ischemia-induced endothelial dysfunction in the forearm [62]. These studies demonstrate that endogenous nitrite is a regulator of the response to ischemia, and that physiological and dietary strategies to increase tissue and plasma nitrite concentration could confer cytoprotection after IR.

Reduction of nitrite

As mentioned above, nitrite can be reduced to NO along a decreasing physiological oxygen and pH gradient. Deoxygenated hemoglobin (deoxyHb) is the predominant nitrite reductase in blood where in the presence of a proton, deoxyHb donates an electron to nitrite to reduce it to NO, resulting in the oxidation of hemoglobin (Reaction 1) [48, 51, 63].

In the absence of other protein targets, NO formed by this reaction binds unreacted deoxyHb to form iron-nitrosyl hemoglobin (Hb-NO) (Reaction 2).

This reaction is regulated not only by pH and hypoxia, but also by the allosteric structural transition of the hemoglobin tetramer. While the oxy relaxed (R) state of hemoglobin has a lower heme reduction potential and is thus a better electron donor, nitrite is more able to bind the deoxy tense (T) state tetramer as its heme center is ligand free. The balance of these two opposing forces results in a maximal rate of nitrite reduction that coincides at the p50 of hemoglobin [48]. Generation of NO by this highly regulated reaction is responsible for the hypoxic vasodilation and lowering of blood pressure observed in healthy human subjects after nitrite infusion or dietary nitrate ingestion respectively [51,62]. A number of nitrite reductase enzymes have been described to mediate nitrite reduction in tissue and these proteins appear to contribute differentially depending on tissue type and oxygen concentration. For example, while xanthine oxidoreductase was first identified as a nitrite reductase in the ischemic heart [64], this reaction requires complete anaerobic conditions to proceed and the amount of bioavailable NO generated by the reaction is unclear due to the fact that the enzyme also generates superoxide, which can rapidly scavenge the NO generated. In addition to xanthine oxidoreductase, cytochrome *c* oxidase [65], cytochrome *c* [47], eNOS, carbonic anhydrase [66] and cytochrome P450 enzymes [67] have also been identified as nitrite reductases.

In the heart, myoglobin has been shown to be a potent nitrite reductase that reacts with nitrite via a reaction identical to that of hemoglobin (Reaction 1) [48-49, 63]. Although homologous in structure to hemoglobin, the monomeric structure of myoglobin renders it “locked” in an R state conformation. Thus, its rate of nitrite reduction (bimolecular rate constant = $12 \text{ M}^{-1}\text{s}^{-1}$ at pH 7 and 37°C) exceeds that of hemoglobin (T state = $0.12 \text{ M}^{-1}\text{s}^{-1}$) [49, 63]. Notably, this reaction appears to play a vital role in nitrite-mediated cytoprotection after myocardial infarction. While low concentrations of nitrite decrease infarct size by 50% after myocardial IR in wildtype mice, nitrite does not mediate cardioprotection in myoglobin deficient mice [68]. Notably, nitrite is cytoprotective in the liver and brain, which do not contain myoglobin. It is unknown which nitrite reductase protein is primarily responsible for cytoprotection in these organs, but it is possible that there is no predominant nitrite reductase in these organs and a number of proteins contribute equally.

The reductive anhydrase reaction

In addition to the nitrite reductase reaction which yields pure bioavailable NO, nitrite may undergo a reductive anhydrase reaction resulting in the generation of N₂O₃. In this reaction one nitrite molecule reacts with deoxygenated hemoglobin to generate NO and methemoglobin as described above. Reaction of another nitrite molecule with methemoglobin yields a species that has Fe²⁺-NO₂[•] like character. NO can react rapidly with this species, resulting in the formation of methemoglobin and N₂O₃ (Reaction 2) [69].

This reaction increases the versatility of nitrite as an ischemic signaling molecule. Through the formation of N₂O₃, which is diffusible and can partition into membranes, nitrite can mediate S-nitrosation of protein targets. During ischemia, this presents a clear advantage over NO, which must be oxidized to NO⁺ to mediate S-nitrosation, since many mechanisms of cytoprotection involve the S-nitrosation of critical protein targets including mitochondrial complex I, calcium channels and caspases.

Evidence of Nitrite-mediated protection after IR

In the last six years, evidence from a number of labs and in several animal models has demonstrated that nitrite mediates cytoprotection after focal IR in four different organs and over a wide temporal window [70-72]. Nitrite has been shown to diminish infarct size in the heart after myocardial IR [58,71,73-74], decrease circulating levels of aminotransferases after hepatic IR [56,71,75], prevent neuronal cell death in the brain after stroke [72] and prevent increases in blood urea nitrogen or creatinine levels after renal IR [70]. Additionally, nitrite has been shown to prevent both neurological and cardiac injury in a model of global IR, cardiac arrest and resuscitation [76], as well as mediate angiogenesis during chronic hindlimb ischemia [52]. In over 10 animal models in which the effects of nitrite were tested in IR, only one study to date has not observed a protective effect of nitrite. In this study intravenous or intraperitoneal administration of nitrite to rats prior to renal IR did not lower IR-induced increases in blood urea nitrogen or creatinine [77]. However, a later study demonstrated that topical administration of nitrite on the kidney prior to IR mediated substantial cytoprotection [70]. Thus, it is reasoned that the route of nitrite administration is central to the degree of protection in renal IR. Beyond animal models, it has recently been demonstrated that dietary nitrite supplementation in the form of beetroot juice attenuated ischemic injury in the forearm [62]. This is the first test of nitrite as a therapeutic for IR injury in humans, although several other trials are currently being planned.

In addition to its ability to protect a number of organs, nitrite works over a large concentration range. Doses as low as 2.4 nmol to a mouse, which elevates plasma nitrite concentration by 200 nM decreases infarct size by 20-67% in a murine model of myocardial infarction [71]. Nitrite mediated cytoprotection appears to demonstrate a biphasic response curve, with low concentrations attenuating tissue damage and this effect diminishing after a critical optimal dose. Notably, this optimal dose is relatively consistent across studies and is approximately 50 nmols administered to a mouse [71] or 500nmols administered to a 10-fold larger rat [72] which results in a plasma nitrite concentration of 10 μM. Interestingly, while NO demonstrates a similar biphasic concentration response in terms of biological action, there is little consensus between studies about the concentration of NO that mediates optimal protection after IR.

Nitrite also confers resistance to ischemia over a wide temporal window. Administration of nitrite to the heart or liver prior to [73,75], during [71] or at a late stage of ischemia prior to reperfusion [78], has been shown to prevent cell death and decrease tissue damage. Importantly, the studies reported were conducted using various routes of administration, including intraperitoneally, intravenously and orally. Further, we have previously shown that nitrite itself mediates delayed pharmacological preconditioning [53]. The administration of oral or intravenous nitrite 24 hours prior to the onset of hepatic or myocardial IR decreases circulating levels of alanine aminotransferase or diminishes infarct size respectively [53]. Remarkably, this phenomenon occurs even though the added nitrite is consumed within 30 min of treatment. While we have proposed that nitrite-dependent S-nitrosation of mitochondrial complex I may play a role in this phenomenon, the mechanism by which nitrite mediates cytoprotection 24 hours after its administration remains unclear. In the following section, the putative mechanisms by which nitrite and/or NO mediates cytoprotection will be discussed.

Potential Mechanisms of Cytoprotection

While the exact mechanism by which nitrite confers resistance to IR injury is unclear, several studies suggest that nitrite reduction to NO is required for the cytoprotective effects. Though the direct measurement of NO in these physiological systems is technically difficult, multiple investigators have directly and indirectly assessed nitrite-dependent NO formation in ischemic organs. For example, Zweier and colleagues first directly measured xanthine oxidoreductase catalyzed nitrite reduction to NO in the acidic ischemic heart by EPR and chemiluminescence [64]. These studies were confirmed by Webb and colleagues who showed increased NO formation and formation of iron-nitrosylated proteins in an isolated Langendorff heart model of IR [73]. In vivo, Okamoto and colleagues first utilized N-15 labeled nitrite to measure the production of N-15 labeled iron-nitrosyl hemoglobin (Hb-NO) by EPR in a model of renal IR [79-80]. Rassaf and colleagues have detected NO generation in the ischemic heart by performing similar experiments. Notably, in these studies nitrite-dependent NO formation is significantly decreased in myoglobin deficient hearts, corroborating that myoglobin is the predominant nitrite reductase in the ischemic heart [81]. In addition to these studies in which NO formation was directly measured, a number of the focal IR studies mentioned above have utilized the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO) [52-53,71,73]. Administration of PTIO prior to or during ischemia inhibited nitrite-mediated cytoprotection. These data suggest that reduction of nitrite, either by the reductase or anhydride reaction to NO or N₂O₃ respectively, is requisite for its cytoprotective action after IR.

In the following section we consider the putative mechanisms that have been described for NO-mediated cytoprotection and discuss the evidence for nitrite-dependent modulation of these pathways. Although several pathways are known to be regulated by NO in the context of IR, discussion of all these pathways (reviewed in [3]) is outside the scope of this article. However, a number of studies provide evidence that modulation of mitochondrial function underlies the protective mechanism of IPC. Further, although the role of nitrite in metabolism is still emerging, NO is a well-characterized regulator of all aspects of mitochondrial function. Thus, here we focus on the mitochondrial targets for NO and/or nitrite that are known to be of importance in determining cytoprotection after IR.

Modifications of mitochondrial proteins

Regulation of ROS through complex I and III

Post-translational modification of respiratory complexes account for some of both the acute and delayed protective effects of NO. Complex I is particularly susceptible to ischemic damage and is a major target for NO and its metabolites. Several studies demonstrate that S-nitrosation of critical thiols on the complex can inhibit the enzyme, resulting in cytoprotection [82-83]. Such modifications by agents such as S-nitrosoglutathione, NO, or S-nitroso-2-mercaptpropionyl glycine (SNO-MPG) [84] have been shown to be cardioprotective in *in vitro* isolated cardiomyocyte models [84], *ex vivo* in isolated perfused hearts, as well as in *in vivo* murine models of myocardial infarction. Inhibition of complex I activity appears to be cytoprotective due to its inhibitory effect on ROS generation, particularly at the time of reperfusion when rapid entrance of oxygen into the electron chain results in a burst of ROS formation. Notably, IPC has also been shown to mediate S-nitrosation of complex I [83]. Given that S-nitrosation is a reversible modification, this complex I inhibition is slowly alleviated over time, allowing for normal electron transport chain activity to resume slowly without the deleterious instigation of oxidative damage. This concept has been termed the “Metabolic Shutdown and Gradual Wake Up” phenomenon and has been attributed to the cytoprotective effects of a number of NO and S-nitrosothiol based pharmacological agents [85]. Indeed, we have reported that nitrite inhibits complex I in several models of IR and that this inhibition decreases hydrogen peroxide generation by the mitochondrion upon reperfusion [53]. Consistent with the gradual wake up hypothesis, this inhibition of complex I activity is fully reversed within 90 minutes of reperfusion [76].

Notably, while acute S-nitrosation at the time of reperfusion decreases ROS generation, inhibition of complex I or III under basal conditions has been reported to increase mitochondrial ROS production [82]. This increase in basal ROS generation has been shown to initiate signaling cascades [86-87] leading to delayed IPC [88]. This signaling cascade, resulting in the activation of genes such as HIF1 α , HO-1 [89] and Hsp60 [90-91] may be implicated in delayed cardioprotection in a manner similar to hypoxia-induced preconditioning [92-93]. Interestingly, it was reported that administration of antioxidant therapy (superoxide dismutase [SOD] plus catalase plus mercaptpropionyl glycine [MPG]) during the preconditioning phase of ischemia abrogated the development of late preconditioning in conscious pigs [94] and MPG alone (which scavenges peroxynitrite [ONOO⁻] and/or hydroxyl radical [•OH]) blocked the initiation of late preconditioning in conscious rabbits [95]. Also, pretreatment with NO donors such as diethylenetriamine/NO (DETA/NO), S-nitroso N-penicillamine (SNAP) and nitroglycerine in the absence of ischemia imparted delayed protection against infarction [96-97]. Again, ONOO⁻ and •OH scavengers terminated these effects. Taken together, these findings suggest that NO mediates late preconditioning against infarction via the formation of ONOO⁻ and/or secondary ROS.

Post-translational modification of ATP synthase

The ATP synthase plays a vital role in cellular energetics as it is the predominant site of ATP formation in the cell. Conversely, this enzyme can work in reverse to catalyze the hydrolysis of ATP. Several studies have now reported that increased ATP hydrolysis by the ATP synthase during ischemia contributes to the depletion of the ATP pool [98-99]. Thus, downregulation of the enzymatic activity of the ATP synthase may be beneficial through the preservation of cellular ATP. Consistent with this mechanism, IPC as well as several pharmacological preconditioning agents, including diazoxide, adenosine, and GSNO, have been demonstrated to regulate ATP synthase activity through the post-translational modification of the protein complex [100-102]. Several types of modifications have been

reported to modulate the ATP synthase in different conditions including phosphorylation, acetylation, and trimethylation (recently reviewed in [103]). With regard to NO signaling, it has recently been reported that the ATP synthase alpha subunit shows increased nitration after IR[104]. However, it is unclear whether nitration plays a regulatory role or is an indicator of oxidative damage to the enzyme in this case. More pertinent to IPC is the finding by Sun and colleagues that GSNO-dependent preconditioning induced a concentration dependent S-nitrosation of the F1-ATP synthase alpha subunit, which inhibited the activity of the complex [102]. These data are consistent with the protective effect of NO and suggest that the NO or nitrite dependent preservation of ATP in the ischemic tissue may contribute to the cytoprotective mechanism of IPC.

S-nitrosation of other mitochondrial proteins

Accumulating evidence demonstrates that S-nitrosation plays a critical regulatory role in the modulation of enzymatic function. While both complex I and the ATP synthase have been identified and investigated as targets for S-nitrosation within the mitochondria, it is likely that S-nitrosation of other proteins within the organelle contributes to IPC-dependent cytoprotection. In the past five years multiple groups have taken a proteomic approach to identify mitochondrial proteins post-translationally modified by IPC and pharmacological preconditioning [102,104-105]. Using this approach, Sun and colleagues recently showed that the overall levels of S-nitrosation are increased in hearts subjected to IPC [102]. Moreover, using GSNO as a preconditioning agent they identified several sites of S-nitrosation beyond the ATP synthase including aconitase, alpha-ketoglutarate dehydrogenase, creatine kinase, malate dehydrogenase, heat shock protein 60 and aldehyde dehydrogenase. Early studies suggest that modification of some of these targets may be important in cytoprotection. For example, GSNO-dependent S-nitrosation of a-ketoglutarate dehydrogenase results in an increase in its enzymatic activity[102]. Taken together with prior studies that demonstrate that IPC prevents an IR-mediated inhibition of a-ketoglutarate dehydrogenase activity, these data are consistent with a protective role for NO-dependent S-nitrosation of a-KDG contributing to the protective mechanism of IPC[102]. While the role of S-nitrosation in the regulation of these newly identified protein targets remains unclear, studies are ongoing to determine whether these modifications play a protective role in IPC and IR.

NO induced glutathionylation

While much focus has been centered on S-nitrosation as a mediator of cytoprotection, the role of glutathionylation, the covalent modification of a cysteine residue with glutathione, in ischemic signaling has recently come to the forefront. While several oxidative mechanisms lead to glutathionylation, it is now clear that nitrosative species, in the presence of GSH also mediate this modification. Mechanistically, GSNO may react directly with a reduced protein yielding a glutathionylated protein (Eq 3) [106-108]:

Additionally, it has been suggested that GSH may react with an S-nitrosated protein to form a protein mixed disulfide (Eq 4)[106]:

Many protein targets within the mitochondria, including complexes I[109] and II[110], and aldehyde dehydrogenase [106], are known to be glutathionylated. While multiple labs have demonstrated that glutathionylation of specific protein sites modulate mitochondrial

function, the role of this modification in IR and cytoprotection remains unclear. It has been posited that glutathionylation, a reversible modification, may serve to temporarily protect susceptible protein thiols from irreversible oxidation (formation of sulfenic, sulfinic or sulfonic derivatives). Consistent with this protective effect, Eaton and colleagues have shown global increases in glutathionylation after administration of IPC[111]. Other studies demonstrate that glutathionylation may mediate more site specific signaling. For example, Chen and colleagues have shown that glutathionylation of complex II is decreased after myocardial infarction and this decrease is accompanied by a loss of enzymatic activity [110]. These data suggest that glutathionylation may be requisite for normal complex II function and also support a protective role for the modification. Notably, many thiols are targets for both S-nitrosation and glutathionylation such as the 75kD subunit of complex I [112-113]. While both modifications inhibit complex I activity, recent studies suggest that they may differentially modulate ROS production by the complex and have differing extents of reversibility [112-113]. Ongoing studies will decipher the role of NO-mediated glutathionylation in cytoprotection and determine whether this modification contributes to the protective effect of IPC.

Nitrosylation of complex IV

Cytochrome *c* oxidase is known to be the predominant target for NO within the mitochondria. Nanomolar concentrations of NO bind both the reduced and oxidized forms of the enzyme in competition with oxygen, leading to the reversible inhibition of oxygen consumption [114-116]. Inhibition of complex IV has consequences for both ROS generation as well as the production of ATP. While binding of NO to complex IV has been demonstrated to increase ROS generation due to electron accumulation at complexes I-III in the electron transport chain [117], this small increase in ROS generation may not play a large role in the progression of injury during ischemia. On the other hand, several investigators have hypothesized that inhibition of complex IV may have major cytoprotective consequences for the preservation of high energy phosphate stores during IR and may underlie the phenomenon of “myocardial hibernation”. In this phenomenon metabolic activity of the heart is temporarily shut down during ischemia in order to preserve high energy phosphate reserves [68]. Indeed, studies have demonstrated that nitrite mediates complex IV inhibition in the heart through the myoglobin dependent generation of NO. However, the protective effect of nitrite is lost in the absence of myoglobin, suggesting that inhibition of complex IV may be a dominant mechanism in this model [49,68].

NO reaction with ubiquinol

Other mitochondrial respiratory chain proteins that have been found to react with NO include Ubiquinol (UQH₂) [118]. Within the cell, ubiquinone (UQ), which predominantly exists in its reduced form UQH₂, is present in the inner mitochondrial membrane, but is also found in plasma membranes, and in the cytosol [119]. Extramitochondrial UQH₂ exerts a strong antioxidant effect by reacting with high concentrations of ONOO⁻ and NO₂⁻ [120]. UQ formed from this reaction is then reduced back to UQH₂ by NADPH-ubiquinone reductase [121]. Biologically, while the reactions of UQH₂ with ONOO⁻ and NO₂⁻ effectively decrease the propagation of oxidative damage caused by nitrogen free radicals, NO scavenging by UQH₂ may decrease NO bioavailability. The effects of NO on mitochondrial UQH₂ are dependent on the O₂ and NO concentration gradients. In normoxia, steady state levels of UQH₂ and NO are estimated as 1.08×10^{-4} M and 1×10^{-7} M, respectively [118]. The reaction between both free radicals (1.73×10^{-7} M.s⁻¹) is favored at high concentrations of UQH₂, such as those elicited when NO inhibits cytochrome *c* oxidase [122] or binds to the cytochrome b region [88]. Binding of NO to cytochromes aa3 and b increase H₂O₂ formation in an antimycin-like manner.

The Mitochondrial K⁺_{ATP} channel

The mitochondrial K⁺_{ATP} channel (mito-K⁺_{ATP}) is a putative ATP-sensitive K⁺ channel that has been purported as an effector of delayed ischemic preconditioning [22,123-124], even though its identity remains controversial [125-126]. Channel openers, most notably diazoxide, were reported to protect against myocardial ischemic injury in various experimental models, while channel blockers such as 5-hydroxydecanoate (5HD) and glibenclamide did not prevent injury [127-128]. However, the specificity of these pharmacological compounds for mito-K⁺_{ATP} has been questioned, as diazoxide is also an inhibitor of complex II [129-130] and 5HD is also a β -oxidation substrate [131]. In spite of these caveats, opening of the mito-K⁺_{ATP} has been implicated in NO-mediated cytoprotection against IR injury. Several mechanisms are thought to mediate this process.

It has previously been reported that NO can selectively activate mito-K⁺_{ATP} channels and a recent study demonstrates that other reactive nitrogen species, including the nitrated lipid nitrolinoleate, nitroxyl (HNO/NO⁻), and S-nitrosothiols can also activate these channels [132]. Both cGMP-independent and dependent mechanisms have been reported for NO-dependent opening of the mito-K⁺_{ATP} channels [133-135]. It has been proposed that cGMP activates Protein Kinase G (PKG), causing the phosphorylation of an unknown target protein which in turn activates Protein Kinase C ϵ (PKC ϵ) and leads to the phosphorylation of the mito-K⁺_{ATP} channel [136]. Notably, activation of PKC ϵ has been shown to play a central role in the IPC pathway and opening of mito-K⁺_{ATP} channels has been shown to increase mitochondrial ROS production triggering preconditioning in the heart [137-138]. Interestingly, similarly to diazoxide, nitrolinoleate and nitroxyl have both been shown to inhibit complex II, suggesting that inhibition of this complex may play a role in activation of mito-K⁺_{ATP} channels [132]. Opening of mito-K⁺_{ATP} channels has also been associated with mild membrane depolarization, increased respiration rates, decreased ATP production, release of accumulated Ca²⁺ and matrix swelling, all of which play a role in preventing apoptosis [127-128,139].

Cytochrome c release

A great deal of attention has been focused on the role of NO in regulating the mitochondrial apoptotic pathway, which is initiated by the oxidation of cardiolipin and release of cytochrome *c*, during IR. It is now established that low concentrations of NO can inhibit cytochrome *c* release from the mitochondria. The electron transport protein cytochrome *c* exists in the mitochondria with its heme in a hexa-coordinate arrangement which enables the protein to shuttle electrons between complex III and IV [140-141]. However, in select situations, cytochrome *c* can undergo a shift to a penta-coordinate arrangement which initiates peroxidase activity by this protein. This peroxidase activity is able to oxidize the polyunsaturated lipid cardiolipin (CL) which normally anchors cytochrome *c* to the outer surface of the inner mitochondrial membrane [142]. Oxidation of cardiolipin (ox CL) allows the dissociation of CL from cytochrome *c* [143], leaving cytochrome *c* freely soluble within the intermembrane space and able to be released. One known mechanism by which NO abrogates cytochrome *c* release is through its nitrosylation of the heme in cytochrome *c*, resulting in the inhibition of its peroxidase activity and subsequent oxidation of CL [144]. The release of cytochrome *c* (and of other pro-apoptotic factors) into the cytosol requires outer membrane permeabilization [145], which also appears to be facilitated by oxCL [146-147]. Therefore, nitrosylation of CL-bound cytochrome *c* represents a mechanism through which NO prevents the initiation of the intrinsic apoptotic pathway. Interestingly, penta-coordinate cytochrome *c* has also been shown to reduce nitrite to NO, suggesting a potential mechanism by which nitrite may mediate cytoprotection [47]. Consistent with this idea, it has been shown that nitrite can prevent mitochondrial permeability transition and cytochrome *c* release after IR.

Assembly of the permeability transition pore

The mitochondrial permeability transition pore (mPTP), a non-specific inner mitochondrial membrane channel of unknown identity, contributes to the sequence of events that leads to IR-induced cell death [24,148]. While elevated matrix calcium levels are major triggers of the mPTP opening, other conditions such as oxidative stress and the depletion of adenine nucleotides have been shown to promote pore opening, and subsequent ATP depletion and mitochondrial swelling, and the release of cytochrome *c* and other pro-apoptotic factors [23,149]. In view of this essential role in apoptosis, the mPTP has been explored as a potential therapeutic target for preventing IR injury [150]. Ischemic preconditioning has been shown to inhibit initial mPTP opening in rat hearts reperfused after 30 min global ischaemia [151]. Post-conditioning-mediated cardioprotection is also believed to occur through prevention of the mPTP formation [152].

It has been proposed that the NO-mediated gradual wake-up of the electron transport chain could indirectly prevent mPTP formation by attenuating mitochondrial Ca^{2+} overload and ROS overproduction [148]. Additional potential mechanisms by which NO may avert mPTP opening include the prevention of apoptosis by inactivation of caspase-3 *via* S-nitrosation [153-155], caspase 3-dependent inhibition of Bcl-2 cleavage [153]. Notably, low concentrations of nitrite have also been demonstrated to inhibit calcium-induced opening of the mPTP after ischemia/reperfusion.

Conclusions

The discovery of the phenomenon of IPC-mediated cytoprotection dramatically changed the field of IR by demonstrating that physiological adaptive mechanisms exist after transient ischemia to prevent injury from a future ischemic insult. Additionally, several subcellular targets, whose modulation are central to cytoprotection after IR, have been uncovered through investigation of the IPC pathway. In the two decades since the discovery of IPC, it has become evident that NO and its metabolites play an integral role in the cell signaling cascades that lead to IPC-dependent cytoprotection and that many of these pathways converge at the level of the mitochondrion. While basic research has proven that NO mimics the beneficial effects of IPC, the development of classical NO donors as therapeutics for IR injury has not been overwhelmingly successful. The recent emergence of nitrite as an endocrine reservoir of NO and hypoxic signaling molecule that mediates potent cytoprotection in a number of IR models has led to the consideration of nitrite as an active mediator of IPC and as a putative successful NO-based therapeutic for IR. The ability of nitrite to remain as a stable reservoir until ischemic conditions are encountered, make it an ideal mediator of NO-like signaling that is specifically targeted to ischemic zones. Moreover, the unique reductase and anhydrase chemistry of nitrite enables more versatility in its signaling. Though the mechanism of nitrite mediated cytoprotection remains unclear, current knowledge about this phenomenon suggests that nitrite holds promise as both a physiological regulator of the ischemic response as well as a potential therapy for ischemic disease.

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Figure 1. Nitrite as a potential mediator of IPC

Ischemic preconditioning (IPC) dependent stimulation of NOS generates NO, which is oxidized to stable nitrite in normoxia. During ischemia (hypoxia and acidosis) nitrite is reduced by various nitrite reductase proteins to either NO or N₂O₃. These species mediate signaling within the mitochondrion by S-nitrosating and inhibiting complexes I, V, opening K_{ATP} channels, preventing cytochrome c release, and nitrosylating complex V. NO also prevents assembly of the permeability pore. GSH present in the mitochondrial matrix is able to react with S-nitrosated proteins to mediate glutathionylation. The regulation of mitochondrial function through these mechanisms results in protection from reperfusion injury.

Table 1

Models of Ischemia/Reperfusion in which nitrite has been shown to be protective.

Species	Heart				Brain	Liver	Kidney	Hindlimb	Forearm
	Rat	Dog	Mouse						
Models	Isolated perfused heart (in vitro)	Myocardial Infarction (coronary artery occlusion)	Myocardial Infarction (coronary artery occlusion)	Cardiac arrest and resuscitation	Stroke (cerebral artery occlusion)	occlusion of hepatic artery/portal vein	Bilateral renal ischemia	Chronic ligation of femoral artery	Ischemia induced endothelial dysfunction
Timepoints/Route of Administration	15 min prior to IR	1 hour or 5 min prior to reperfusion	Oral in drinking water i.v. 5 min prior to ischemia oral gavage 24 h prior to ischemia	i.v. prior to resuscitation	i.v. at time of reperfusion i.v. for 7 days after IR	i.p. injection During ischemia i.p. injection 24 hours prior to ischemia oral in drinking water prior to IR	Typically 1 min prior to reperfusion	i.p. injection during chronic ischemia	Oral nitrate prior to study
References	[73,156]	[78]	[53,58,68,71]	[76]	[72,157]	[53,56,71]	[70]	[52]	[62]