

## REVIEW

# Nitric Oxide Mechanism of Protection in Ischemia and Reperfusion Injury

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## ABSTRACT

In 1992 nitric oxide (NO) was declared molecule of the year by *Science* magazine, and ever since research on this molecule continues to increase. Following this award, NO was shown to be a mediator/protector of ischemia and reperfusion injury in many organs, such as the heart, liver, lungs, and kidneys. Controversy has existed concerning the actual protective effects of NO. However, literature from the past 15 years seems to reinforce the consensus that NO is indeed protective. Some of the protective actions of NO in ischemia and reperfusion are due to its potential as an antioxidant and anti-inflammatory agent, along with its beneficial effects on cell signaling and inhibition of nuclear proteins, such as NF- $\kappa$  B and AP-1. New therapeutic potentials for this drug are also continuously emerging. Exogenous NO and endogenous NO may both play protective roles during ischemia and reperfusion injury. Sodium nitroprusside and nitroglycerin have been used clinically with much success; though only recently have they been tested and proven effective in attenuating some of the injuries associated with ischemia and reperfusion. NO inhalation has, in the past, mostly been used for its pulmonary effects, but has also recently been shown to be protective in other organs. The potential of NO in the treatment of ischemic disease is only just being realized. Elucidation of the mechanism by which NO exerts its protective effects needs further investigation. Therefore, this paper will focus on the mechanistic actions of NO in ischemia and reperfusion injury, along with the compound's potential therapeutic benefits.

**Keywords:** Nitric Oxide, Ischemia, Reperfusion, Mechanism of protection

## INTRODUCTION

It has been over 200 years since the discovery of nitric oxide (NO $\cdot$ ) by John Priestly [1], and yet research

on this small molecule is still increasing. In 1988 Bob Furchgott proposed that endothelium-derived relaxing factor might be NO [2], and in 1992 NO was named molecule of the year [3]. Since then many articles have been published regarding its mechanism of action and therapeutic potential, although without complete consensus. NO has been shown to be released by the conversion of L-arginine to L-citrulline, a reaction catalyzed by one of three NO syntheses

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Table 1. Brief review of the chemical and molecular protective effects of nitric oxide.

Effects as	Description	Sources
1. Antioxidant	Free radical scavenger	14, 16
	Inhibits cell respiration	17, 18
	Restores antioxidant enzyme levels	15
2. Anticytokines	Suppresses TNF- $\alpha$	22, 28, 29
	Suppresses IL-1	31
3. Antiadhesion molecules	Inhibits selectins	24, 34
	Inhibits VCAM-1 and ICAM-1	24, 36
4. Antiapoptotic	Downregulates p53 gene	44
	Inhibits human caspases	45, 47
	Induces expression of HSP70	48
5. Antichemokines	Downregulates MIP-1 and MIP-2	51, 54
6. Antidetrimental signaling	Regulates MAPKs	56, 58
	Promotes preconditioning	59
	Produces elevated levels of cGMP	60
7. Antinuclear proteins	Inhibits NF- $\kappa$ B	63–66
	Inhibits AP-1	68

(NOS): neuronal NOS, endothelial NOS, and inducible NOS [4, 5]. Furthermore, NO has been shown to be a mediator/protector of ischemia and reperfusion (I/R) tissue-mediated injury [6]. NO has been shown to be tissue-protective through its physiologic regulation of vascular tone, inhibition of platelet aggregation, attenuation of leukocyte adherence to the endothelium, scavenging of oxygen-derived free radicals, maintenance of normal vascular permeability, inhibition of smooth muscle proliferation, immune defenses, and stimulation of endothelial cell regeneration [7]. Delivery of NO during an ischemic insult has been shown to limit the extent of reperfusion damage to the heart [8], liver [9], lungs [10], and kidneys [11]. In the past there has been debate concerning whether NO is protective. However, the majority of literature from the past 15 years demonstrates that NO is indeed protective, and that the dose and timing of administration may have caused controversy [8, 12, 13] (Tables 1 and 2). This paper focuses on the mechanistic actions of NO in I/R injury and the compound's potential therapeutic benefits.

## CHEMICAL AND MOLECULAR EFFECTS OF NITRIC OXIDE

### Nitric Oxide as an Antioxidant

NO has been shown to have antioxidant properties not related to alterations in neutrophil migration or adhesion, which may account for much of the protective effect of NO during I/R injury. The antioxidant properties of NO in tissues may be related to the reduction by NO of superoxide anion-mediated tissue toxicity [13]. NO reacts with the superoxide anion to form peroxynitrite, which may act as a superoxide radical scavenger [14]. This action doesn't allow peroxynitrite to perpetuate the lipid peroxidation chain reactions, which would result in the generation of other free radicals, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxide [15]. Furthermore, NO acts as an oxygen radical scavenger [16]. NO has also been shown to inhibit mitochondrial respiration [17, 18], and thereby reduces the generation of reactive oxygen species after I/R injury, which can contribute to cellular injury, necrosis, and apoptosis. NO appears to modulate cell respiration by inhibiting cytochrome-c oxidase before ischemia, which can create an adaptive preconditioning environment [19]. NO competes with oxygen for binding to cytochrome-c oxidase, so the interaction of NO with the electron transport chain is more pronounced when there is less oxygen, as in ischemia. When reperfusion occurs, ischemia will slowly be relieved, preventing abrupt resumption of the electron transport chain, and generation of less reactive oxygen species [12]. The conversion of H<sub>2</sub>O<sub>2</sub> and myoglobin to ferryl myoglobin has also been shown to be prevented by NO, and this action may prevent the initiation of lipid peroxidation [20]. Kawachi and associates observed eNOS-deficient mice subjected to a period of ischemia. They found that eNOS-derived NO modulates I/R-induced injury to the liver. Their model, however, did not involve the infiltration of polymorphonuclear neutrophils, so it may have involved enhanced reactive oxygen species-dependent injury [21]. Rodriguez-Peña and associates also demonstrated that molsidomine, an NO donor, administered 15 min before reperfusion, prevented an increase in superoxide anion due to I/R [22]. Additionally, it has been shown that pretreatment of rats with molsidomine restores the depleted renal antioxidant enzymes, such as glutathione, catalase, and superoxide dismutase [15].

### Anti-Inflammatory Effect of Nitric Oxide

Nitric Oxide has been found to be a mediator of many inflammatory processes, including the prevention of neutrophil infiltration [23] and the reduction of pro-inflammatory cytokines [24]. These anti-inflammatory responses are essential for the protection against

Table 2. Selective review of the literature pertaining to the anti-ischemic and anti-inflammatory actions of NO.

Authors	Type of anti-inflammatory action	Animal type and ischemia time	Treatment	Results
López-Neblina et al., 1995 [91]	Less lipid peroxidation	Rat, 75 min	SNP, 60 min prior to ischemia	Kidney injury protective effect
López-Neblina et al., 1996 [11]	Prevention of neutrophil infiltration	Rat, 75 min	SNP at 75, 30, 15, and 5 min prior to reperfusion	Kidney injury protective effect
Murakami et al., 1997 [10]	Reduced PMN sequestration	Rat, 60 min	Inhaled NO, during ischemia, at reperfusion 15 min after reperfusion	Lung injury protective effect
Massoudy et al., 2000 [78]	Less activated leukocytes and platelets	Human	SNP, 60 min of reperfusion	Heart injury protective effect
Martinez-Mier et al., 2000 [44]	Diminished apoptosis	Rat, 75 min	SNP, 15 min before reperfusion	Kidney injury protective effect
Aiba et al., 2001 [92]	Improved hepatic microcirculation	Dog, 60 min	FK409, intravenously for 30 min	Liver injury protective effect
Martinez-Mier et al., 2002 [51]	Downregulated MIP-1 and MIP-2	Rat, 75 min	SNP, 15 min prior to reperfusion	Kidney injury protective effect
Cakir et al., 2003 [93]	Reduced neutrophil infiltration	Dog, 120 min	SNP, after release of aortic cross-clamp	Lung injury protective effect
Anaya-Prado et al., 2003 [94]	Reduced cytokine expression	Rat, 90 min	SNP, at 30 min after bleeding began	Hemorrhagic shock protection
Rodríguez-Peña et al., 2004 [22]	Reduced pro-inflammatory cytokines	Rat, 60 min	Molsidomine, 15 min before reperfusion	Kidney injury protective effect
Kuroki et al., 2004 [77]	Increased hepatic microcirculation	Rat, 60 min	SNP, continuously during reperfusion	Kidney injury protective effect
Chander et al., 2005 [15]	Restored antioxidant enzymes	Rat, 45 min	L-arginine, 30 min before reperfusion and molsidomine, 30 min before ischemia, 12 h after reperfusion	Kidney injury protective effect
Garreffa et al., 2006 [95]	Coronary vasodilation	Rat, 1.5 h	SNP, 30 min before and throughout metabolic inhibition	Heart injury protective effect
Liu et al., 2007 [87]	Reduced leukocyte infiltration	Pig, 50 min	Inhaled NO, 10 min before balloon deflation and through reperfusion	Heart injury protective effect
Chattopadhyay et al., 2008 [89]	Reduced neutrophil infiltration	Rat, 60 min	L-arginine, 6 days prior to ischemia/reperfusion	Liver injury protective effect

tissue I/R injury (Table 2). Next, we will introduce the molecular inflammatory protection given by NO.

### Nitric Oxide as an Anti-TNF and Anti-IL-1 Agent

Nitric Oxide has been shown to mediate the expression of tumor necrosis factor (TNF), a well-known cytokine, which seems to induce the accumulation

of neutrophils by indirect mechanisms involving the macrophage, and induces the production of other cytokines [22, 25] (Figure 1). Upon reperfusion, TNF- $\alpha$  has been shown to act as a continuous stimulant for polymorphonuclear neutrophil infiltration in the liver, a critical event in I/R injury [26]. TNF- $\alpha$  has also been suggested to stimulate chemokine synthesis in ischemic tissues, and to activate NF- $\kappa$ B, a transcription factor involved in the regulation of genes related to the inflammatory response [27]. NO donors administered

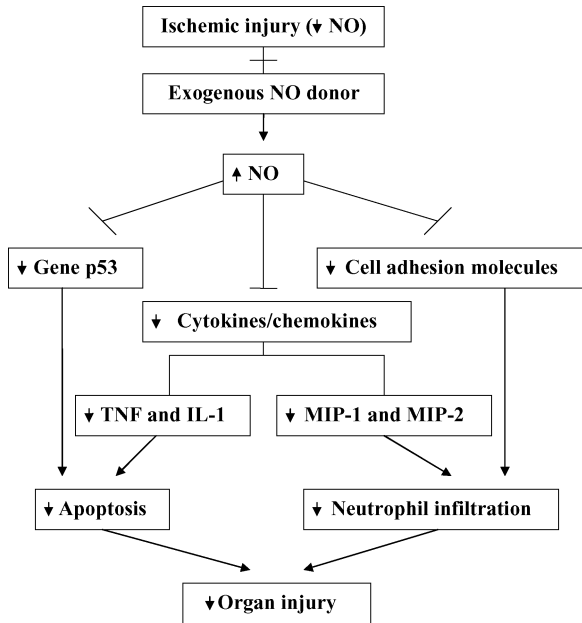


Figure 1. Possible mechanistic effects on the role of ischemic injury and exogenous NO in organ protection. During ischemic injury there is a downregulation of NO in the cell. However, if an exogenous NO donor is administered during the ischemic insult, NO concentrations can be elevated. This elevated level of NO may be able to inhibit gene p53 activity, decrease levels of pro-inflammatory cytokines and chemokines, as well as decrease levels of cell adhesion molecules. Specifically, TNF- $\alpha$ , IL-1, MIP-1, and MIP-2 are the cytokines and chemokines which are reduced by NO. Through the suppression of gene p53 and lowered levels of TNF- $\alpha$  and IL-1 in the cell, the amount of apoptosis can be reduced. And through the suppression of cell adhesion molecules and lowered levels of MIP-1 and MIP-2  $\alpha$ -chemokines, less neutrophil infiltration can occur. Decreased apoptosis and neutrophil infiltration help to protect the cell during ischemia and reperfusion and lead to a decrease in organ injury.

during the ischemic event appear to suppress TNF- $\alpha$  [28]. The effect of direct intrarenal administration of molsidomine, a known NO donor, on renal dysfunction and inflammatory reaction after I/R was investigated by Rodriguez- Peña et al. They measured the levels of pro-inflammatory cytokines 48 h after blood flow re-establishment and found that treatment with molsidomine significantly lowered the levels of TNF- $\alpha$ . They determined that molsidomine exerted protective effects on renal function when administered during the ischemic period. This protective action seemed to be mediated, in part, by the reduction of pro-inflammatory cytokines, such as TNF- $\alpha$  [22]. Anaya-Prado and associates investigated the effects of another NO donor, nitroprusside, on the lung after I/R injury. The effect of this NO donor on pro-inflammatory cy-

tokines was measured by ribonuclease-protection assay, and they found that the expression of all cytokines, including TNF- $\alpha$ , was reduced by sodium nitroprusside [29]. Hatano et al. have investigated the effect of a NO donor, S-nitroso-N-acetylpenicillamine (SNAP), on TNF- $\alpha$ -mediated apoptosis. TNF- $\alpha$  moderated cell death in iNOS (-/-) mice demonstrated the protective actions of NO against TNF- $\alpha$ -mediated apoptosis [30]. The expression of another cytokine, interleukin-1 (IL-1), also appears to be mediated by NO. IL-1 is a pro-inflammatory cytokine, which can contribute to the deleterious effects of I/R injury [26]. Franco-Gou and associates demonstrated that preconditioning through NO inhibits IL-1 release, and this is the method by which NO exerts its protective effects against hepatic I/R [31].

### Nitric Oxide as an Anti-Adhesion Molecule

The expression of cell adhesion molecules (CAMs), such as the selectins, is also influenced by NO. After I/R injury, CAMs are upregulated in the cell due to activation induced by a variety of inflammatory molecules, including cytokines and chemokines [32]. Selectins have been shown to mediate the initial attachment between polymorphonuclear neutrophils and activated endothelium, a requisite step in the I/R injury model [33]. P-selectin plays a role in the early phase of rolling and adherence of leukocytes in the microvasculature after ischemia reperfusion. Our lab has shown that P-selectin (-/-) animals were more protected than controls at 3 h after ischemia, confirming the key role of P-selectin in liver I/R injury. Furthermore, I/R has been shown to produce a decrease in NO levels and an increase in P-selectin levels in the cell [34]. This increase in P-selectin levels may be due to superoxide and H<sub>2</sub>O<sub>2</sub> levels in the cell. It has also been demonstrated that infusion of a NO donor decreased leukocyte rolling and adherence, and prevented P-selectin expression on the surface on the venular endothelium [35]. De Caterina and associates have demonstrated the effects of NO on cytokine-induced expression of effector molecules characteristic of endothelial activation. Using three different NO donors they showed that NO inhibits other cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and endothelial-leukocyte adhesion molecule-1 (E-selectin) [24]. The long-term effect of NO on cell adhesion molecules has also, more recently, been demonstrated by Waldow et al. They demonstrated that SNAP administration reduced the induction of VCAM, ICAM, and E-selectin by the chemokines TNF- $\alpha$  and IL-1 $\beta$  [36]. Jiang et al. also investigated the cell surface and mRNA expression of adhesion

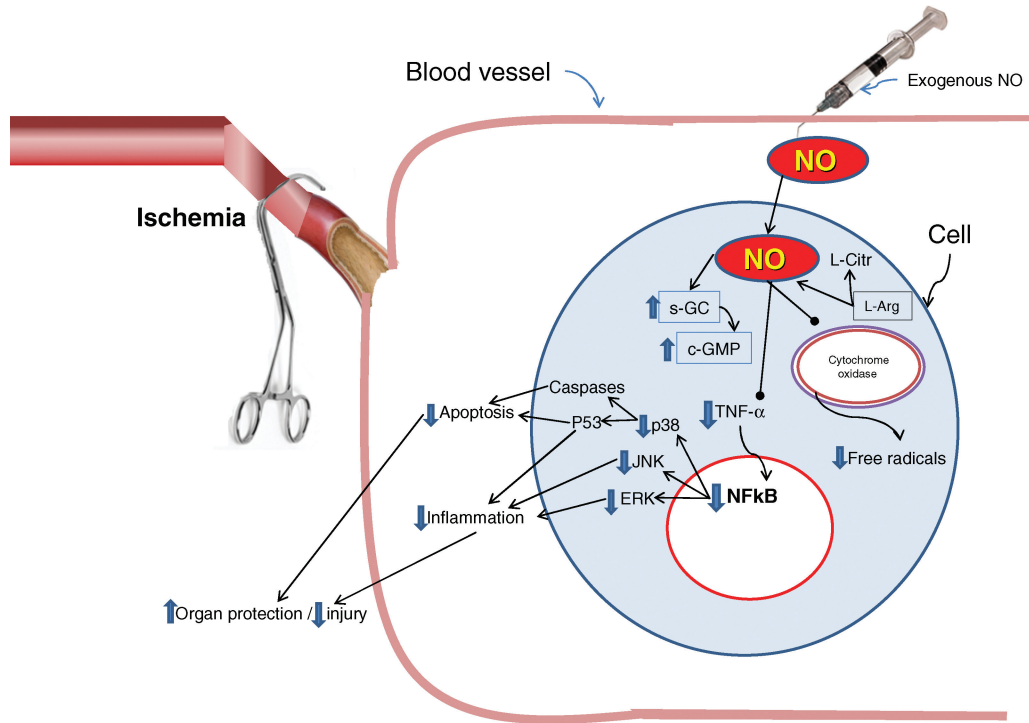


Figure 2. NO and its effects on cell-signaling pathways. A clamp can be applied to the blood vessels to experimentally induced ischemia in cells. During this induced ischemia an exogenous NO donor can be administered into the blood. This NO then migrates into the cell where it can afford protection during ischemia. NO can also be endogenously released in the cell by the conversion of L-arginine to L-citrulline. Once in the cell NO gives protection through many pathways. NO can inhibit cytochrome oxidase in the mitochondria to reduce the amount of free radicals produced. NO can also inhibit TNF- $\alpha$ , which goes on to inhibit NF- $\kappa$ B. NF- $\kappa$ B can then inhibit MAPKs, which include p38, ERK, and JNK. The downregulation of p38 can then lead to the inhibition of caspase-3 and gene p53. Overall, the downregulation of p53, ERK, and JNK can lead to a decrease in cell inflammation. NO is also protective through its stimulation of sGC, which goes on to increase cGMP levels in the cell. cGMP has the potential to inhibit caspase-3 activity, which would otherwise be necessary for apoptosis. The decrease in cell inflammation and apoptosis due to elevated levels in the cell can lead to an increase in organ protection during ischemia and reperfusion.

molecules, E-selectin, ICAM-1, and VCAM-1, in human dermal microvascular endothelial cells exposed to the NO donor, spermine NONOate. They found that these CAMs, which are induced by TNF- $\alpha$ , are reduced upon treatment with spermine NONOate, possibly by blocking redox-regulated NF- $\kappa$ B activation [37].

### The Anti-Apoptotic Effect of Nitric Oxide

Different mechanisms cause NO to exhibit a proapoptotic or antiapoptotic effect [38, 39] (Figure 2). However, the amount of apoptosis induced by TNF- $\alpha$ , oxidative stress, and serum or glucose deprivation has been shown to be decreased by NO. When there are high NO concentrations in the cell, necrosis and apoptosis occur, such as when cells are exposed to high levels of exogenous NO donors [40, 41]. Nonetheless, application of a NO donor in some cells, such as hepato-

cytes, human B-lymphocytes, endothelial cells, splenocytes, eosinophils, and PC12 cells, inhibits apoptosis [42]. This may be due to a diminished response of guanylyl cyclase to NO, although the mechanism by which NO inhibits apoptosis may vary between cell types [42, 43]. NO in our lab has also been shown to inhibit apoptosis by downregulating the expression of gene p53, which normally promotes apoptosis in the kidneys. Animals with worse histopathological features, after I/R injury, were found to have elevated levels of p53 gene expression. From this study we confirmed the beneficial effects of an exogenous NO donor during an ischemic insult and demonstrated that the downregulation of gene p53 correlated with a decrease in apoptosis [44]. Furthermore, NO has been demonstrated to dose-dependently inhibit recombinant human caspases, a family of cysteine proteases that play a critical role in the initiation and execution of apoptosis [45]. The caspase cascade activation is required for p53-dependent apoptosis [46].

The ability of NO to inhibit caspases [47] is due to S-nitrosylation of the cysteine thiol in the presence NO. In addition, NO has been shown to induce the expression of heat shock proteins, such as HSP70, which have been depicted to protect hepatocytes from apoptosis [41]. Kim and associates demonstrated the effect of NO on HSP70. They found that NO is able to induce HSP70, and that this induction protected rat liver cells from TNF- $\alpha$  apoptosis [48]. The influence of inhaled NO on apoptosis induced after I/R has been investigated by Yamashita et al. Inhaled NO during reperfusion greatly attenuated the increase of TUNEL-positive cells in ischemia-reperfusion. They demonstrated that inhaled NO attenuated apoptosis after pulmonary I/R injury [49]. Appropriate amount of NO seem to suppress unwanted apoptosis [50].

### **Nitric Oxide as an Anti-Chemokine: MIP-1 and MIP-2**

NO has been shown to regulate the chemokine response, which includes macrophage inflammatory proteins MIP-1 and MIP-2. These chemokines have been shown to act as neutrophil chemoattractants [25]. Ischemia and reperfusion injury is dependent on neutrophil infiltration, so MIP-1 and MIP-2 contribute to the detrimental effects of reperfusion. Moreover, NO can regulate the chemokine response through a series of signaling pathways, potentially those associated with MAPK, SAPK/JNK, and JAK/STAT [51]. Candrasekar and associates demonstrated that I/R induces the expression of MIP-2, and that MIP-2 was present in infiltrating cells [52]. Our lab investigated the role that exogenous NO, from the NO donor sodium nitropruside, plays on the regulation of MIP-2 and MIP-1  $\alpha$ -chemokines. The relationship between NO and MIP-2 and MIP-1  $\alpha$ -chemokines was confirmed, and we noticed that NO downregulated the production of these chemokines after I/R injury [51]. Recently the role of NO in MIP-2 production has been investigated by Shibata and associates. They first demonstrated that ERK1/2 is involved in MIP-2 production [53]. Then they showed that NO suppressed activation of ERK1/2 and NF- $\kappa$ B [54]. Walpen and associates also investigated the relationship between NO and MIP-2. They exogenously administered DETA-NO, an NO donor, to rat mesangial cells, and contradictory to what we saw, observed an increase in MIP-2 expression. They then determined that NO produced by neutrophils and mesangial cells has the potential to amplify leukocyte recruitment [55]. This may have been due to the dose given, timing, or the cell environment in which the NO donor was administered.

### **Anti-Detrimental Signaling of Nitric Oxide**

Nitric Oxide appears to be an important modulator of mitogen-activated protein kinases (MAPKs). MAPKs can transduce signals into cellular responses by modulating gene and protein expression involved in cell differentiation, proliferation, survival, and death [38]. Some of the MAPKs include extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinases [45, 56]. These MAPKs are activated due to early events of I/R, including the activation of TNF- $\alpha$  and other proteins [57]. ERK, p38 MAPK, and JNK have been shown to be activated by NO-related species and to participate in NO signal transduction [56]. Nitric Oxide influences downstream MAPK activation through the nitrosylation of critical thiol residues, which possibly activates specific protein kinase C (PKC) isoforms. The post-translation modification of low molecular weight G proteins is the mechanism by which NO may signal independent of PKC [58]. NO has been shown to promote beneficial signaling during preconditioning, a process which can protect organs from I/R injury. Carini and associates investigated the mechanism by which NO exerts beneficial effects. They concluded that NO can induce preconditioning of hepatocytes by promoting the sequential activation of guanylate cyclase, cGK, and p38 MAPK [59]. A recent study by Wang et al. sought to investigate the role of p38 MAPK signal transduction pathway on apoptosis induced by NO. They demonstrated the p38 signal transduction pathway of NO-induced articular chondrocyte apoptosis in a rabbit model. Furthermore, they showed that p38 is able to stimulate NF- $\kappa$ B, p53, and caspase-3 activation, which can lead to chondrocyte apoptosis [40]. It has also been suggested that MAPKs can intensify inflammatory signaling, and that the appropriate inhibition of MAPKs could potentially help ameliorate the ischemic response [57]. NO has also been shown to activate sGC, which leads to an elevation of cyclic GMP (cGMP). cGMP, through downstream effectors, has been shown to regulate neurotransmission, cell migration, proliferation, differentiation, survival, axon outgrowth and guidance, and visual signal transduction [60]. One of these mechanisms regulated by cGMP may be the process by which NO exerts beneficial signaling during I/R injury.

### **Anti-Nuclear Protein Effect of Nitric Oxide**

Nitric Oxide has been shown to inhibit both NF- $\kappa$ B and AP-1 transcription factors, which have been shown to play important roles in I/R injury. NF- $\kappa$ B has been shown to be activated by cytokines, such as TNF- $\alpha$  [57], and by free radicals. Furthermore, the genes regulated by this transcription factor include those involved in the inflammatory response, cell adhesion, and

apoptosis [27, 61]. The genes regulated by NF- $\kappa$ B include iNOS, TNF- $\alpha$ , IL-1, and IL-6 [62]. NO has been shown to inhibit NF- $\kappa$ B activation, possibly through the induction of I $\kappa$ B- $\alpha$  [63] or by nitrosylating a redox-active cysteine residue in NF- $\kappa$ B that would attenuate binding to DNA. It is also possible that the inhibition of NF- $\kappa$ B by NO occurs because of its scavenging of the superoxide radical, which could otherwise activate NF- $\kappa$ B [64, 65]. Matthews and associates investigated the ability of an NO donor to influence DNA binding activity of NF- $\kappa$ B. They used sodium nitroprusside and S-nitroso-N-acetylpenicillamine and found that NO donors could inhibit the DNA binding of NF- $\kappa$ B. They proposed that NO donors inhibited NF- $\kappa$ B by stabilizing I $\kappa$ B $\alpha$  and increasing the transcription of I $\kappa$ B $\alpha$  genes [66].

Nitric Oxide has also been demonstrated to inhibit AP-1 activity. AP-1 contributes to post-I/R injury, due to its possible involvement in intracellular signaling pathways leading to apoptosis, or via its involvement in signaling events triggered by cytokines, such as TNF- $\alpha$  [67]. Tabuchi and associates investigated the effects of an NO donor, sodium nitroprusside, on the transcription factor AP-1. They concluded that cells administered SNP-experienced attenuation of AP-1. Potassium ferrocyanide, an analogue of SNP devoid of NO, was unable to inhibit the increase in tissue plasminogen activator (TPA)-responsive element-binding activity induced by kainite. This failure of potassium ferrocyanide supports the involvement of NO in AP-1 attenuation [68].

## Therapeutic Potential of Nitric Oxide

The literature amply demonstrates that following I/R there is a deficit of NO in the cell. Thus, it seems plausible, and has been shown in the heart [8], liver [69], lungs [70], and kidneys [71] that administration of NO during I/R may help to attenuate subsequent injury.

## Exogenous Nitric Oxide Donors

Nitric Oxide donors, such as nitroprusside and the organic nitrate nitroglycerin, have been used clinically with success. Sodium nitroprusside is mainly used to treat hypertensive emergencies; however, it can also be used when short-term reduction of cardiac preload and/or afterload is desired [72]. Cyanide toxicity is a concern in the administration of sodium nitroprusside, although it is relatively uncommon in the surgical intensive care unit [73], and may be avoided by combination with another drug, such as trimetaphan or a  $\beta$ -adrenergic receptor blocker [74]. On the other hand, nitroglycerin has many more therapeutic options, as in

the treatment of angina, congestive heart failure, unstable angina pectoris, non-ST-segment-elevation myocardial infarction, acute myocardial infarction, and variant angina [72]. Tolerance to nitroglycerin can occur with long-term usage, but the vitamins C or E, which are antioxidants, along with the organic nitrate therapy have been shown to prevent tolerance to the drug [75]. Our lab, in 1995, demonstrated the beneficial effects of nitroprusside on the ischemically damaged rat kidney, concluding that exogenous NO is beneficial and protective to the kidney during I/R [76]. These beneficial effects have more recently been demonstrated again by Kuroki and associates, who administered nitroprusside to the rat kidney during reperfusion and found increased hepatic microcirculation and reduced hepatocyte damage [77]. We also investigated the time dependence of sodium nitroprusside administration in the rat ischemic kidney. Administration of sodium nitroprusside occurred at 75, 30, 15, and 5 min before reperfusion. We concluded that sodium nitroprusside could be administered as late as 15 min before reperfusion and still be effective in the rat ischemic kidney [11]. NO donors have also been used experimentally with success to improve cardiac [8, 78], pulmonary [70, 79], and liver [22, 80] I/R injuries. Many NO donors, other than nitroprusside and nitroglycerin, exist and each demonstrates different modes and rates of NO release. These include spontaneously releasing NO in solution, releasing NO over a prolonged period of time at steady rates, or releasing NO in specific tissues [81, 82]. Therefore, an NO donor may be specifically chosen for the disease it will treat [3].

## Direct Nitric Oxide Donor: NO Inhalation

Inhaled NO has been used in the past because of its pulmonary effects, but its possible therapeutic use as a mediator of I/R injury is being investigated [75]. The clinical applications of inhaled NO include persistent pulmonary hypertension of the newborn [83], bronchopulmonary dysplasia, and pulmonary hypertension in adults [84]. Barbotin-Larrieu and associates showed that, in a neonatal piglet model of lung I/R, inhaled NO could prevent microvascular injury, endothelial dysfunction, and pulmonary neutrophil accumulation. Thus, inhaled NO prevented some of the reperfusion-induced injury [85]. The timing and concentration of inhaled NO are vital to its protective effects, and Murakami et al. have investigated these factors. They found that I/R injury is best attenuated by NO at 30 ppm given immediately at reperfusion or 15 min after the beginning of reperfusion [10]. Schutte and associates have also shown that inhaled NO induces a state of preconditioning and maintains endothelial integrity in subsequent I/R [86]. Inhaled NO has mostly

been studied in the ischemic lung; however, it has been shown to be effective in other organs as well. The effect of NO after myocardial I/R was investigated by Liu et al. They showed improvement in microvascular perfusion and reduction of infarct size after inhalation of NO just before and during coronary reperfusion [87]. Inhaled NO following orthotopic liver transplantation has, very recently, been investigated by Lang et al. They demonstrated that inhaled NO at 80 ppm, given to patients undergoing orthotopic liver transplantation, significantly lowered hepatocyte apoptosis, thereby reducing the injury related to I/R [88]. Inhaled NO has been shown numerous times to be effective in models of I/R and even in human clinical trials, again demonstrating the effectiveness of exogenously administered NO.

### Endogenous NO: L-Arginine

L-arginine is an endogenous source of NO, which is released during the conversion of L-arginine to L-citrulline. Administration of L-arginine can be protective to ischemic organs. Chander and associates investigated the effects of L-arginine administration in I/R-induced renal failure in rats. The L-arginine administration occurred 30 min before the renal ischemia, and pretreatment with this drug attenuated renal dysfunctions and morphological alterations, improved the tissue as well as urine NO contents, reduced elevated thiobarbituric acid reactant (TBAR) levels and restored depleted renal antioxidant enzymes [15]. Chattopadhyay and associates have also presented results that support the protective function of L-arginine in rat liver I/R. They administered L-arginine, 100 mg/kg body weight/daily for 7 days before induced I/R, and found heightened NO production in hepatocytes along with diminished hepatocellular injury in rats that received this treatment. They concluded that L-arginine was protective in rat liver I/R, and that it was protective due to increased production of NO [89]. Furthermore, we investigated the effects of L-arginine in the lung after hemorrhagic shock and resuscitation. Our findings showed that L-arginine was protective to the injured lung due to reduction in acute lung injury observed in the histological studies [29]. However, a more recent study by Rusai et al. in the ischemic rat kidney, observed that L-arginine (2 g/kg body weight daily) didn't affect the injury sustained from I/R, but did increase the mRNA expression of NOS isoforms. This may be explained by time and dose administration differences between studies, along with the severity of the ischemic injury [90]. Again, more studies examining the ideal dose and time of administration of L-arginine will be helpful in determining its possible therapeutic value.

### CONCLUSION

The mechanisms by which NO exerts protective effects in I/R injury have eluded discovery for many years. We are only just beginning to understand the complex mechanism of action of NO. It protects against I/R injury due to its potential as an antioxidant and anti-inflammatory agent, as well as its beneficial effects on cell signaling and its inhibition of nuclear proteins. Consensus is being reached in the debate regarding an NO protective effect, with most studies reporting its protective effects. The mechanism of this protection requires further investigation. More therapeutic possibilities for NO continue to be suggested, including the potential of NO as an agent to attenuate I/R injury. The ideal time and dose of NO administration need to be clearly delineated so that clinical trials of this drug can be performed, and its potential in preventing I/R injury can be reached.

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