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Review

Nitric oxide in myocardial ischemia/reperfusion injury

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Abstract

Administration of nitric oxide (NO), NO donors or drugs that enhance NO realease (statins, calcium antagonists, ACE-inhibitors, dexamethasone) prior to ischemia protects the myocardium against ischemia/reperfusion injury. While this exogenous administration of NO prior to ischemia can initiate a preconditioning-like phenomenon, endogenous NO-synthase (NOS)-derived NO is not involved in triggering or mediating the early phase of ischemic preconditioning's protection, but does play a pivotal role for initiating and mediating the delayed phase of ischemic preconditioning's protection.

The present review now summarizes the importance of endogenous and exogenous NO when given at the time of reperfusion for vascular and myocardial function and morphological outcome following ischemia/reperfusion. Given the inconsistency of the published data, potential confounding factors that might affect experimental results on the role of NO in myocardial ischemia/reperfusion were identified, such as (1) the lack of characterization of the involved NOS isoforms in myocardial ischemia/reperfusion injury in different animal species, (2) the lack of direct measurements of myocardial NO concentration and/or NOS activity to assure sufficient NOS inhibition, (3) the lack of consideration of nonenzymatic NO production as a potential source of NO, and (4) the absence of plasma or blood components in in vitro studies influencing NO delivery and metabolism.

Future research on the importance of NO in ischemia/reperfusion injury will have to focus more precisely on the identification and standardization of potential confounding experimental factors that influence synthesis, transport, and interaction of NO with various targets in blood and tissue.

Keywords: Nitric oxide; Infarct size; Stunning

1. Introduction

Administration of nitric oxide (NO) or NO donors prior to ischemia attenuates the consequences of myocardial ischemia/reperfusion; i.e., reduces infarct size and endothelial dysfunction [1]. These beneficial effects of NO are related to a pharmacological type of preconditioning, and the existing literature has been reviewed extensively by Roberto Bolli [1]. Also, pretreatment with drugs that enhance NO release such as statins [2], certain calcium antagonists [3], ACE inhibitors [4] or dexamethasone [5] protects the myocardium against ischemia/reperfusion injury. While exogenous administration of NO prior to ischemia can initiate a preconditioning-like phenomenon, endogenous NO-synthase derived NO is not involved in triggering or mediating the early phase of ischemic preconditioning's protection (for review, see Ref. [6]), but does play a pivotal role for initiating and mediating the delayed phase or second window of ischemic preconditioning's protection (for review, see Ref. [7]). To what extent NO is involved in the pharmacologically induced second window of protection is still a matter of debate (for review, see Ref. [8]).

Given the above established facts, the present review will concentrate (1) on the importance of endogenous NO for vascular and myocardial function in myocardial ischemia/reperfusion, and (2) on the importance of exogenous NO when given at the time of reperfusion for the functional and morphological outcome following myocardial ischemia/reperfusion, and (3) to identify potential confounding factors

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that might affect experimental results on the role of NO in myocardial ischemia/reperfusion and which could explain some of the inconsistency of the results obtained so far.

2. Production of nitric oxide

Nitric oxide synthases (NOS) are the enzymes responsible for NO generation. To date, three distinct NOS isoforms have been identified: neuronal NOS (type 1), inducible NOS (type 2) and endothelial NOS (type 3). NOS's catalyze an overall 5-electron oxidation of one N^{ω}-atom of the guanidino group of L-arginine to form NO and L-citrulline, with the intermediate N^{ω}-hydroxy-L-arginine (NOHA). NO synthesis is critically influenced by various cofactors such as tetrahydrobiopterin, flavin mononucleotide and flavin adenine dinucleotide, the presence of reduced thiols, the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) and, of course, substrate availability (Fig. 1). Also, NOS1 and NOS3 are dependent on calmodulin and calcium [9]. Without an adequate delivery of substrate and co-factors, NOS no longer produces NO but instead transfers the free electrons to oxygen and thus produces free oxygen radicals [10].

Under resting conditions, NO synthesis has been mainly attributed to the vascular endothelium and its constitutively active NOS3. Both NOS1 and NOS3 have been identified in cardiomyocytes, and their expression appears to be species-dependent in that NOS1 is more important in rats while NOS3 is of greater importance in rabbits [11]. The expression of NOS differs within the left ventricular wall of ferrets and humans, with a higher expression in the subepicardium than in the subendocardium [12], and also the subcellular distribution of NOS 1 and NOS 3 within cardiomyocytes differs, with NOS3 present in caveolae located at the outer membrane and NOS1 located at the sarcoplasmic reticulum [13]. Given its distinct location, NOS3 has been proposed to mainly interact with β-adrenoceptors and L-type calcium channels, thereby attenuating calcium influx into the cardiomyocyte [14]. Conversely, NOS1 has been proposed to interact with ryanodine receptors, thereby increasing calcium release from the sarcoplasmic reticulum [15]. Platelets and leukocytes also carry NOS isoforms [16,17]; however, those NOS isoforms contribute significantly to NO formation only upon activation [18,19].

The NOS3 activity is increased by phosphorylation of its serine residue 1177; this phosphorylation is achieved by activation of PI3-kinase and protein kinase B (Akt). In contrast, phosphorylation at the threonine residue 495 by AMP-activated kinase or protein kinase C can inactivate NOS3 [13] (Fig. 1).

The cardiac interstitial NO concentration is within the nanomolar range during normoperfusion [20]. During ischemia, NOS3 activity is increased within minutes [21], and subsequently the NO concentration during early ischemia is increased [22]. However, with prolonged myocardial ischemia, NOS3 protein expression decreases [23,24], and the

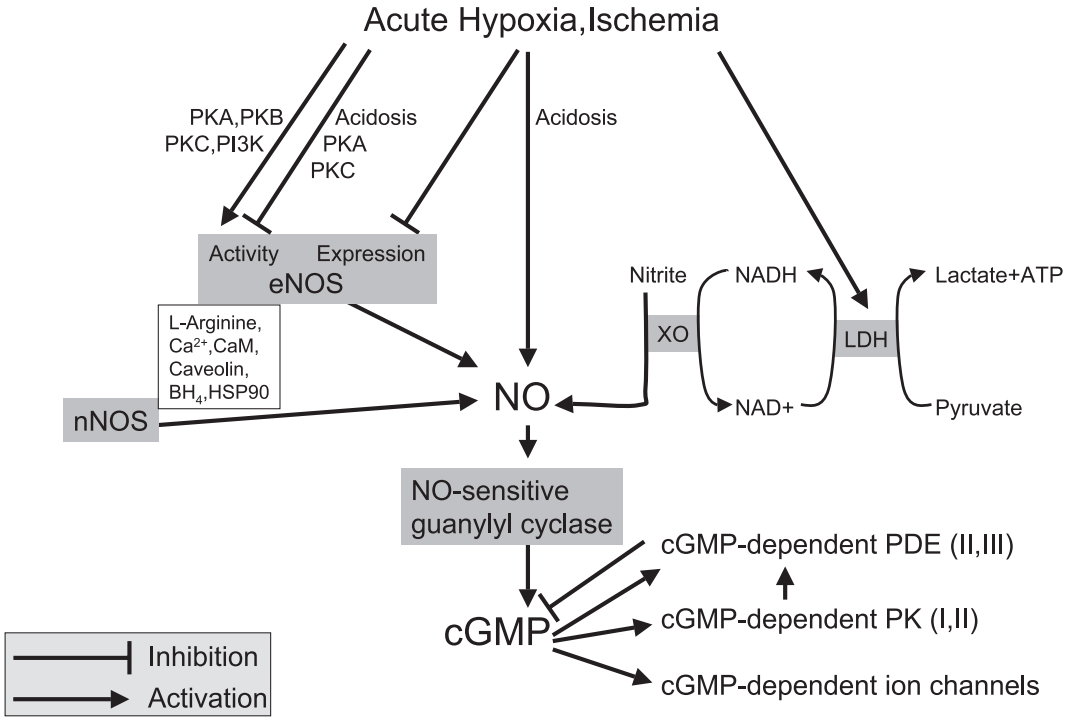

Fig. 1. Factors contributing to NO production and downstream targets of NO. For details, see text. PKA: protein kinase A, PKB: protein kinase B, PKC: protein kinase C, PI3K: phosphoinositol-3 kinase, CaM: calmodulin, BH_4: tetrahydrobiopterin, HSP90: heat shock protein 90, XO: xanthine oxidase, LDH: lactate dehydrogenase, PDE: phosphodiesterase, PK: protein kinase.

increased tissue acidosis attenuates NOS3 activity[24]. Within the early seconds of reperfusion, the NO concentration is again increased [23]; however, with prolongation of reperfusion NOS activity and thus NO concentration decrease below baseline values [25]. Also, neurohumoral factors affect NO availability. Angiotensin II, which is increased in concentration during myocardial ischemia [26], is capable of increasing NOS3 protein expression; however, since—at the same time—free radical production is increased, the myocardial NO concentration is decreased [27]. Tumor necrosis factor (TNF)α, which is rapidly released within the ischemic myocardium [28–30], decreases NOS3 protein expression by reducing eNOS mRNA stability [18], but induces NOS2 protein expression in leukocytes, however not in cardiomyocytes [31].

Apart from NOS-derived NO, a non-enzymatic production of nitric oxide exists during ischemia [32]. Tissue acidosis occurring during ischemia increases NO production independent from NOS3 [33], and even at normal pH, xanthine oxidase in the presence of low pO_2 and high NADH concentration is capable of producing NO from nitrite [34]; the nitrite concentration in plasma amounts to 0.5 μM and in vascular tissue up to 10 μM [35]. Indeed, in isolated rat heart [22] and in rabbit hindlimb muscle [36], the NO concentration is still increased during ischemia after complete NOS inhibition by L-NNA.

In conclusion, the cardiac interstitial NO concentration during early ischemia and early reperfusion is increased. The increase in the NO concentration is in part derived from activated NOS isoforms (species-dependent differences) but also from NOS-independent pathways.

3. Metabolism of nitric oxide in the mammalian circulation

The charge neutrality of NO facilitates its free diffusion in aqueous solutions and across cell membranes. The biological effects of NO are dependent on its half-life, which depends on the rate of NO formation (see above) and its metabolism. In principle, NO can react by electron gain to form the nitroxyl anion (NO^-) and by electron loss to form the nitrosonium ion (NO^+). Various metabolic routes and reactions contribute to the breakdown and/or conversion of NO, NO^- and NO^+, e.g., heme proteins such as guanylyl cyclase, catalase, xanthine oxidase, superoxide dismutase (SOD) and hemoglobin, or high-energy free radicals such as the hydroxyl radical or carbon, oxygen- and nitrogen-centered radicals [10].

The major immediate breakdown product of NO in plasma is nitrite (NO_2^-) [9]. Nitrite can be taken up by red blood cells, where it is oxidized in a hemoglobin-dependent manner to nitrate (NO_3^-), which can subsequently be redistributed into plasma. NO also rapidly interacts with superoxide anions to produce the potent oxidant peroxynitrite ($ONOO^-$). The formation of free oxygen radicals is increased during reperfusion, depending on duration and severity of the preceding ischemia; thus with more severe and prolonged ischemia free radical formation during the subsequent reperfusion is increased [37], subsequently also increasing the formation of peroxynitrite. High concentrations of peroxynitrite are thought to oxidize thiols or thioethers, to nitrate tyrosine residues, to nitrate and oxidize guanosine, to degrade carbohydrates, to initiate lipid peroxidation, and to cleave DNA. The peroxynitrite in excess decomposes to yield NO_3^-.

Alternatively, NO can react with O_2 to yield reactive intermediates. It is well appreciated that the autooxidation of NO in an aqueous environment leads to the formation of reactive NO species such as dinitrogen trioxide (N_2O_3). This intermediate can nitrosate as well as oxidize different substrates to yield either *N*-nitroso or *S*-nitroso compounds. Recent data provide unequivocal evidence for nitrosative chemistry of NO in human plasma. Redox-active thiols, which are abundantly present in plasma, can incorporate NO and transport it throughout the mammalian circulation in the form of bioactive nitrosothiols [38].

In conclusion, depending on its environment (buffer, plasma, blood, high pO_2, presence of free oxygen radicals) NO forms different reactive intermediates which dose-dependently react with surrounding tissue components.

4. NO-cyclic guanosine monophosphate (cGMP) pathway

The major target of NO in the cardiovascular system is the NO-sensitive guanylyl cyclase or soluble guanylyl cyclase (for review, see Ref. [39]) (Fig. 1). Activation of the guanylyl cyclase results in the conversion of guanosine triphosphate to the second messenger cGMP. CGMP activates two specific cGMP-dependent protein kinases (PKG I and II), PKG I being most important for vasodilation and inhibition of platelet aggregation (for review, see Ref. [40]). CGMP also inhibits the activity of phosphodiesterases (PDE II and III); inhibition of PDE III elevates the concentration of cyclic adenosine monophosphate (cAMP), thereby subsequently increasing the activity of protein kinase A (PKA; for review, see Ref. [41]). A low concentration of cGMP appears to primarily inhibit PDE III, while a higher cGMP concentration activates PKG [39–41].

During ischemia, the ensuing acidosis reduces the guanylyl cyclase activity in isolated rat cardiomyocytes [42], thereby potentially counterbalancing the increased NO concentration during early ischemia. Indeed, the myocardial cGMP concentration did not increase significantly during 40 min of low flow ischemia in isolated buffer-perfused rat hearts [43] and 90 min low flow ischemia in in situ pig hearts [44]. In contrast, the cGMP concentration was increased in ischemic areas compared to normoperfused areas of patients with coronary artery disease [45,46]. Following ischemia/reperfusion, however, myocardial cGMP concentration was decreased compared to baseline in isolated rat hearts as well as in pig hearts in vivo [47].

In conclusion, the cardiac cGMP concentration during ischemia is possibly somewhat increased, while upon reperfusion it is clearly decreased. This time course of the alteration in cGMP concentration matches that of the cardiac NO concentration. Dose-dependently, cGMP inhibits PDE or activates PKG, thereby mediating its effects on the vasculature, platelets and myocytes.

5. Coronary vascular effects of NO

Pharmacological inhibition of endogenous NO synthesis increases blood pressure [44,48,49], and lack of NOS3 causes mild hypertension in mice [50]. Also in the presence of a coronary stenosis, NO contributes to the maintenance of regional myocardial blood flow [51]. The NO-induced vasodilation results from a decreased intracellular calcium concentration in vascular smooth muscle cells secondary to direct activation of calcium-dependent potassium channels, cGMP-dependent activation of PKGI and cGMP-dependent inhibition of voltage-gated calcium channels (for review, see Ref. [40]). Apart from its direct vasodilatory effect, NO can preserve myocardial perfusion by inhibiting platelet aggregation and leukocyte adherence to the vascular endothelium (Fig. 2), the latter most likely independently from cGMP. NOS activity is reduced during reperfusion [25], as evidenced also by loss of NO-dependent vasodilation in response to acetylcholine [52] or bradykinin [53] or loss of vasoconstriction in response to NOS inhibition [54], and neutrophil adherence to the endothelium progressively increases during reperfusion [55]. Preservation of endothelial function by administration of L-arginine [56], a NO donor [57], or low concentrations of peroxynitrite (1–2 μM) [58] inhibits such neutrophil adherence and their subsequent infiltration following ischemia/reperfusion. Following ischemia/reperfusion, myocardial infiltration of mononuclear cells contributes to irreversible tissue injury, since pharmacological blockade of NOS2—which is located in the mononuclear cells but not in cardiomyocytes—reduced infarct size following 30 min coronary artery occlusion and 48 h reperfusion in rabbits [59].

Endogenous NO also attenuates the ischemia-induced increase in adenosine. Following NOS inhibition with L-NAME, the myocardial adenosine concentration during ischemia is increased in isolated rabbit hearts [60]. The effect of NOS inhibition on the myocardial adenosine concentration is mediated via protein kinase C and subsequently 5′ nucleotidase [61,62].

6. Inflammation and NO

Both adenosine and TNFα are involved in ischemia/reperfusion injury [6,63]. Endogenous NO either directly or through an altered myocardial adenosine concentration [64] facilitates the ischemia-induced increase in the myocardial TNFα concentration; accordingly, NOS inhibition completely abolishes the increase in the myocardial TNFα

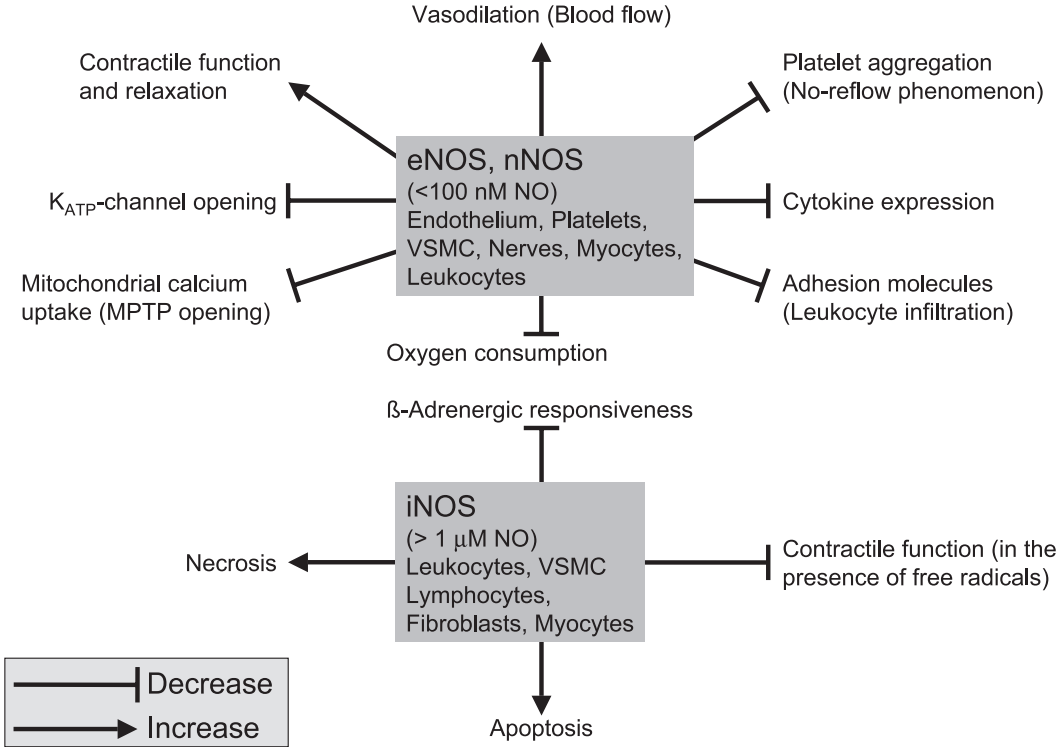

Fig. 2. Effects of low (eNOS- or nNOS-derived) or high NO (iNOS-derived) concentrations. VSMC: vascular smooth muscle cells, MPTP: mitochondrial permeability transition pore.

concentration secondary to coronary microembolization in dogs [65].

7. Cardiomyocyte function

As mentioned above, NO via cGMP dose-dependently inhibits PDE and/or activates PKG. At low NO/cGMP concentrations (in nM range), inhibition of PDEIII activity or direct activation of adenylyl cyclase [66] with subsequently increased cAMP concentration and PKA activity increases cardiomyocyte function (for review, see Ref. [41]). Additional mechanisms by which low NO/cGMP concentrations might increase cardiomyocyte function relate to a direct activation of ryanodine receptors or voltage-operated calcium channels [13]. Indeed, pharmacological blockade of NOS3 in pigs in vivo reduced regional myocardial function for a given blood flow and oxygen consumption, supporting a positive inotropic effect of the basal endogenous NO concentration [44]. Loss of NO release following ischemia/reperfusion (see above) could therefore contribute to the loss of postischemic contractile function.

At higher NO/cGMP concentrations (in μM range), activation of PKG inhibits voltage-dependent calcium channels [13,41] and decreases myofilament calcium responsiveness by phosphorylation of troponin I [67]. While a higher NO/cGMP concentration also suppresses the increase of regional myocardial function during β-adrenergic stimulation [68], most likely by directly inhibiting ryanodine receptors, pharmacological blockade of endogenous NOS-dependent NO synthesis in pigs did not impact on β-adrenergic responsiveness [69]. Thus, only at high concentrations might NO/cGMP directly reduce cardiomyocyte function.

8. Cardiomyocyte mitochondria and NO

Endogenous NO interacts with mitochondrial respiration, possibly at several steps of the electron transfer. In isolated rat hearts subjected to 20 min ischemia and 20 min reperfusion, myocardial ATP content was increased following NOS inhibition, and the production of free oxygen radicals was reduced [70]. Furthermore, in isolated mitochondria, exogenous NO (4 μM) when administered together with calcium increased the production of peroxynitrite in the presence of an unaltered respiration, supporting a shift from oxygen usage for ATP production towards the production of free oxygen radicals [71].

A low concentration of the NO donor SNAP (2 μM) increases the mitochondrial membrane potential via activation of mitochondrial ATP-dependent potassium channels [72]. Any increase in the mitochondrial membrane potential will decrease the mitochondrial calcium uptake, and indeed exogenous NO reduced mitochondrial calcium overload during simulated ischemia [73].

9. NO and apoptosis

In isolated cardiomyocytes and hearts, high NO concentrations (μM range) induce necrosis and apoptosis [74,75]. The amount of necrosis and apoptosis critically depends on the energetic situation of the cardiomyocytes, with apoptosis favored at preserved ATP pools [76]. While the development of necrosis following NO application appears to be independent of cGMP, the development of apoptosis involves cGMP and subsequently activation of mitogen-activated protein kinases and transcription factors [74,77,78]. Most interestingly, the development of apoptosis following application of a NO donor is decreased, once cardiomyocytes or isolated hearts have been subjected to a preceding period of ischemia/reperfusion [74,75], possibly by a diminished response of guanylyl cyclase to NO.

In conclusion, NO can preserve ischemic blood flow and attenuate platelet aggregation and neutrophil–endothelium interaction following ischemia/reperfusion. Low concentrations of NO also increase cardiomyocyte function. On the contrary, higher NO concentrations depress cardiomyocyte function, mediate inflammatory processes following ischemia/reperfusion, impair mitochondrial respiration and even induce cardiomyocyte death. Thus, NO mediates protective as well as deleterious myocardial effects which are critically dependent on the specific experimental conditions.

10. Considerations on confounding variables in studies on NO effects

When trying to define the effects of NO on the heart following ischemia/reperfusion, the results are clearly affected by the experimental preparation and setup:

1. Addition of red blood cells to an isolated buffer perfused heart preparation reduces the extent of irreversible tissue injury by delivering NO to the myocardium [79].
2. Addition of neutrophils to an isolated buffer-perfused heart preparation worsens the functional and morphological outcome following ischemia/reperfusion [55], since neutrophil adherence to the vascular endothelium is increased following ischemia/reperfusion [54]. This increased neutrophil adherence is related to loss of NO production during reperfusion. Preservation of endothelial function by administration of L-arginine [56] or a NO donor [57] inhibits neutrophil adherence following ischemia/reperfusion.
3. Administration of heparin to in vivo preparations affects the morphological outcome following ischemia/reperfusion, since heparin abolishes platelet aggregation and subsequently plugging of small vessels [80] and thereby counteracts the adherence and aggregation otherwise seen following a decrease in NO concentration.
4. The ischemia/reperfusion-induced free radical production differs between in vitro buffer-perfused hearts and in situ

hearts of anesthetized or awake animals, resulting in a substantially different production of peroxynitrite during reperfusion.

5. Species-dependently different NOS isoforms contribute to a different extent to ischemia/reperfusion injury. In rats, the contribution of NOS1 to ischemia/reperfusion is greater than that of NOS3, while in rabbits the major isoform involved in ischemia/reperfusion injury is NOS3 [11].
6. The time and duration of NOS inhibitor administration (bolus application of a high NOS inhibitor concentration vs. continuous administration of a low NOS inhibitor concentration) lead—even within the same animal species —to opposite effects [81]. This observation is

Table 1
Effects on functional recovery following myocardial ischemia/reperfusion

Study	Species	Model	Ischemia (min)	Reperfusion (min)	Inhibitor	Dose	Dose	Effect on myocardial function	Comments
[84]	Rabbit	In vitro	60 (LF)	30	L-NMMA		1 μM	DP ↑	Dose-dependency
					L-NMMA		100 μM	DP↔	Decreased baseline DP
[20]	Rabbit	In vitro	120 (LF)	70	L-NNA		100 μM	DP↔	
					L-NMMA		30 μM	DP↔	
[85]	Dog	In vivo	10 (RI)	360	L-NNA	360 μg/kg	~ 21 μM	WT ↓	
[48]	Dog	In vivo	15 (RI)	240	L-NAME	20 mg/kg	~ 960 μM	WT↔	
[86]	Rat	In vitro	27 (GI)	40	L-NOARG		10 μM	DP ↓	
[87]	Rat	In vitro	30 (GI)	30	L-NAME		100 μM	DP ↓	
[88]	Lamb	In vitro	120 (GI)	30	L-NAME		1 mM	DP ↓	Cold cardioplegia added
[89]	Rat	In vitro	37 (GI)	20	L-NNA		25 μM	DP ↑	Decreased baseline DP
[90]	Rabbit	In vitro	35 (GI)	30	L-NAME		3 μM	DP ↑	
[91]	Rat	In vitro	35 (GI)	30	L-NAME		100 μM	DP ↓	
[92]	Rat	In vitro	60 (GI)	45	L-NAME		1 mM	DP ↑	
[55]	Rat	In vitro	25 (GI)	45	L-NAME		250 μM	DP ↓	PMN added to perfusate
[93]	Rat	In vitro	60 (GI)	60	L-NAME		100 μM	DP ↓	
[94]	Rat	In vitro	60 (GI)	60	L-NAME		100 μM	DP↔	
[95]	Rat	In vitro	120 (GI)	40	L-NAME		100 μM	CO ↓	Cold cardioplegia added
[96]	Guinea pig	In vitro	15 (GI)	35	L-NOARG		1 μM	EHW↔	
[97]	Rat	In vitro	40 (GI)	30	L-NAME	10 mg/kg	~ 480 μM	DP↔	
[98]	Rat	In vitro	20 (GI)	30	L-NMMA		10 μM	DP ↑	Dose-dependency
					L-NMMA		1 or 100 μM	DP ↓	
[99]	Rat	In vitro	25 (GI)	25	L-NOARG		1 mM	DP ↓	
[100]	Dog	In vivo	15 (RI)	120	L-NAME	20 μg/kg/min	~ 140 μM	WT ↑	Given at reperfusion
[70]	Rat	In vitro	20 (GI)	20	L-NAME		0.1, 0.5, 1 mM	DP↔	
[22]	Rat	In vitro	30 (GI)	30	L-NNA		4.6 μM	DP↔	
[101]	Mice	In vitro	16 (GI)	60	L-NAME		100 μM	DP ↓	
[102]	Rat	In vitro	25 (GI)	30	L-NAME		50 μM	DP↔	
[103]	Guinea pig	In vitro	20 (GI)	20	L-NMMA		30 μM	DP ↓	
[104]	Dog	In vivo	20 (RI)	20	L-NNA	4.8 mg/kg	~ 285 μM	WT ↑	
Knockout mice									
[105]	Mice	In vitro	16 (GI)	60	NOS3 −/−			DP ↓	
[106]	Mice	In vitro	20 (GI)	30	NOS3 −/−			DP ↓	
Administration of NO or NO donors just before or at the time of reperfusion									
[107]	Dog	In vivo	15 (RI)	180	NO-gas		70%	WT ↓	
[108]	Rat	In vitro	35 (GI)	30	GS-NO		20 μM	DP ↑	
[109]	Rat	In vitro	15 (RI)	35	L-Arg		3 μM	DP ↓	
[110]	Rabbit	In vitro	30 (RI)	20	SNAP		0.01, 1, 100 nM	DP ↓	Erythrocytes added
[111]	Rat	In vitro	30 (GI)	60	NOO_2^-		800 nM	DP ↑	
[112]	Rat	In vitro	30 (GI)	60	SIN-1		100 μM	DP ↓	
[99]	Rat	In vitro	25 (GI)	40	SNP		1, 10, 100 μM	DP ↑	
[100]	Dog	In vivo	15 (RI)	120	L-Arg		1 mM	WT ↓	
[113]	Rabbit	Myocytes	20 (SI)	30	c-GMP		1 μM	Shortening ↓	
[114]	Mice	In vitro	20 (GI)	30	NOS3 over-expression			DP ↑	

(LF): low flow ischemia; (RI): regional ischemia; (GI): global ischemia; (SI): simulated ischemia; DP: left ventricular developed pressure; WT: regional myocardial function assessed as wall thickening; CO: cardiac output; EHW: external heart work; PMN: polymorphonuclear neutrophils.
L-NMMA: N^{ω}-methyl-L-arginine acetate; L-NNA, L-NOARG: N^{ω}-nitro-L-arginine; L-NAME: N^{ω}-nitro-L-arginine methylester. HCl; $NOS3^{-/-}$: NOS3 knockout; GS-NO: *S*-nitrosoglutathione; L-Arg: L-arginine; SNAP: *S*-nitroso-*N*-acetyl-D,L-penicillamine; NOO_2^-: peroxynitrite; SIN1: 3-morpholinosydnonimine; SNP: sodium nitroprusside; CGMP: cyclic guanosine monophosphate; ↑: increased;↔: unchanged; ↓: decreased.

in part explained by a dose-dependent selectivity of NOS inhibitors for the different NOS isoforms.

7. NOS inhibitors such as L-NAME or L-NNA in the presence of vitamin C can release NO at a rate of 1:200 to 1:1000 of the administered inhibitor concentration [82]. Thus, administration of millimolar concentrations of L-NAME or L-NNA may result in the release of micromolar NO concentrations, thereby potentially substituting for the achieved NOS inhibition.
8. Gene knockouts for NOS1/NOS3 in mice to establish their effects in ischemia/reperfusion injury might also be counteracted by compensatory increases in other proteins such as NOS2 [83]. Moreover, using gene chips it was demonstrated that in NOS1 and NOS3 knockout mice 67 and 47 genes, respectively, were expressed differentially compared to the respective wild type mice. Some of the encoded proteins are known to be involved in ischemia/reperfusion injury (e.g., heat shock proteins) [6].

Table 2
Effects on irreversible tissue injury following myocardial ischemia/reperfusion

Study	Species	Model	Ischemia (min)	Reperfusion (min)	Inhibitor	Dose	Dose	Effect on irreversible myocardial injury	Comments
[115]	Rabbit	In vivo	30/50 (RI)	120	L-NAME	10 mg/kg	~ 480 μM	↓	
[81]	Rabbit	In vivo	30 (RI)	48 h	L-NAME	300 μg/kg/min	~ 480 μM	↑	Continuous infusion of L-NAME
[56]	Dog	In vivo	90 (RI)	60	L-NNA		1 mM	↑	Compared to cardioplegia
[116]	Rat	In vitro	30 (GI)	30	L-NAME		100 μM	↑	LDH measured
[117]	Rabbit	In vitro	30 (RI)	120	L-NNA	15 mg/kg	~ 890 μM	↑	
[60]	Rabbit	In vitro	45 (RI)	180	L-NAME		30 μM	↓	Associated with an increase in adenosine; blocked by SPT
[118]	Rat	In vitro	30 (RI)	120	L-NOARG		100 μM	↔	
[119]	Rabbit	In vitro	30 (RI)	120	L-NAME		100 μM	↔	
[22]	Rat	In vitro	30 (GI)	20	L-NNA		4.6 μM	↔	LDH measured
[120]	Guinea	In vitro	20 (GI)	20	L-NAME		10 μM	↑	LDH measured
					L-NMMA		100 μM		
[121]	Rabbit	In vitro	30 (RI)	120	L-NAME		100 μM	↔	
[122]	Pig	In vivo	90 (LF)	120	L-NNA	30 mg/kg	~ 1.78 mM	↔	
[123]	Guinea pig	In vivo	30 (RI)	120	L-NAME		100 μM	↔	
Knockout mice									
[124]	Mice	In vivo	20 (RI)	120	NOS3 −/−			↑	
[83]	Mice	In vivo	30 (RI)	120	NOS3 −/−			↓	Upregulation of iNOS, following blockade of iNOS: Increased
[125]	Mice	In vitro	30 (GI)	30	NOS3 −/−			↑	LDH measured
[126]	Mice	In vivo	30 (RI)	120	NOS3 −/−			↑	Strain-dependency
								↓	Upregulation of iNOS, following blockade of iNOS: Increased
[127]	Mice	In vitro	35 (GI)	30	NOS3 −/−			↔	
Administration of NO or NO donors just before or at the time of reperfusion									
[128]	Cat	In vivo	90 (RI)	270	NO		~ 600 nM/kg/h	↓	
[129]	Feline	In vivo	90 (RI)	270	SIN-1	0.1 mg/kg	4.6 mg/kg	↓	
[130]	Cat	In vivo	90 (RI)	270	L-Arg	30 mg/kg	730 mg/kg	↓	
[57]	Dog	In vivo	60 (RI)	270	SPM5185		500 nM	↓	
[131]	Pig	In vivo	45 (RI)	240	L-Arg	1 mg/kg/min	40 mg/kg	↓	Retroinfusion
[132]	Dog	In vivo	60 (RI)	240	SIN-1C		5 mM	↓	
[133]	Dog	In vivo	90 (RI)	270	CAS1609	1.25 mg/kg	5.7 mg/kg	↓	
[117]	Rabbit	In vivo	30 (RI)	120	L-Arg	25 mg/kg	525 mg/kg	↔	
[109]	Rat	In vitro	65 (GI)	30	L-Arg		3 mM	↔	Cardioplegia added
[112]	Rat	In vitro	30 (GI)	60	SIN-1		100 μM	↑	LDH measured
[58]	Cat	In vivo	90 (RI)	270	NOO_2^-		1 μM	↓	
[134]	Feline	In vivo	90 (RI)	270	NOO_2^-		2 μM	↓	Dose-dependency
							0.2, 20 μM	↔	
[72]	Mice	In vitro	35 (GI)	30	SNAP		1 μM	↓	

(RI): regional ischemia; (GI): global ischemia; (LF): low flow ischemia; LDH: lactate dehydrogenase.
L-NMMA: N^{ω}-methyl-L-arginine acetate; L-NNA, L-NOARG: N^{ω}-nitro-L-arginine; L-NAME: N^{ω}-nitro-L-arginine methylester. HCl; $NOS3^{-/-}$: NOS3 knockout; L-Arg: L-arginine; SNAP: *S*-nitroso-*N*-acetyl-D,L-penicillamine; NOO_2^-: peroxynitrite; SIN1: 3-morpholinosydnonimine; SPM5185,CAS1609: NO donors; ↑: increased; ↔: unchanged; ↓: decreased.

Keeping the above limitations in mind, we now will summarize the existing literature on the importance of NO for the functional and morphological outcome following myocardial ischemia/reperfusion.

11. Importance of NO for contractile function following myocardial ischemia/reperfusion

In NOS3 knockout mice functional recovery following myocardial ischemia/reperfusion is attenuated (Table 1), while over-expression of NOS3 accelerates functional recovery. Also, pharmacological blockade of NOS attenuates the recovery of left ventricular developed pressure following ischemia/reperfusion in isolated mice and guinea pig hearts. Taken together, these studies in mice and guinea pigs suggest that endogenous NO is cardioprotective and attenuates myocardial stunning.

However, studies in other species using different NOS inhibitors at different concentrations did not unequivocally support the above conclusion. In rats, a dose-dependent effect of NOS inhibitors was observed with low (low micromolar concentrations) or high concentrations (>50 μM) being ineffective or even aggravating myocardial stunning while an intermediate concentration of NOS inhibitors (10–20 μM) improved recovery of contractile function following ischemia/reperfusion. Similar findings were obtained in isolated rabbit hearts, in which low concentrations of NOS inhibitors improved functional recovery while high concentrations of NOS inhibitors had no effect. Finally, in dogs, a low concentration of a NOS inhibitor decreased recovery of regional myocardial function, while higher concentrations of NOS inhibitors either improved or had no effect on functional recovery following ischemia/reperfusion.

While exogenous NO in rabbits and dogs always worsened the functional outcome following ischemia/reperfusion, independent of the approach to increase the NO concentration (inhaled NO, L-arginine, SNAP), in rats the functional recovery following ischemia/reperfusion was either decreased or increased without a clear dose-dependency or relation to the NO donor used.

In conclusion, the importance of NO for the functional recovery following myocardial ischemia/reperfusion appears to be species-dependent. While in guinea pigs and mice endogenous NO mediates beneficial effects, blockade of endogenous NO in rabbits and dogs improves and exogenous NO worsens the functional outcome following myocardial ischemia/reperfusion.

12. Importance of NO for irreversible tissue injury following myocardial ischemia/reperfusion

NOS3 knockout mice have increased infarct size following myocardial ischemia/reperfusion; however, in some mice strains blockade of the compensatorily over-expressed NOS2 (using specific NOS2 inhibitors) was necessary to demonstrate the detrimental effect of the absence of NOS3 for ischemia/reperfusion injury (Table 2).

Administration of NO or NO donors shortly before or at the time of reperfusion did not cause adverse effects, but in some instances decreased irreversible tissue injury. Part of the beneficial effect achieved by exogenous NO or NO donors was mediated by attenuated neutrophil adherence to the vascular endothelium [56].

In conclusion, the above data point towards a reduction of irreversible tissue injury by endogenous NO or exogenous NO.

13. Final conclusion

In summary, when reviewing the existing literature we were surprised by its inconsistency: (1) the lack of characterization of the involved NOS isoforms in myocardial ischemia/reperfusion injury among different animal species; (2) the lack of direct measurements of myocardial NO concentration and/or NOS activity to assure sufficient NOS inhibition (given the different concentrations of NOS inhibitors used); (3) the lack of consideration of non-enzymatic NO production as a potential source of NO; (4) and the absence of plasma or blood components in in vitro studies which impact on the one hand on NO delivery and metabolism and on the other hand on myocardial perfusion, thus making a direct comparison of studies impossible.

Future research in this field will have to focus more precisely on the identification and standardization of potential confounding experimental factors that influence synthesis, transport, and interaction of NO with various targets in tissue and blood.

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