

# Dual modulation of nitric oxide production in the heart during ischaemia/reperfusion injury and inflammation

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## Summary

Nitric oxide (NO) homeostasis maintained by neuronal/endothelial nitric oxide (NO) synthase (n/eNOS) contributes to regulate cardiac function under physiological conditions. At the early stages of ischaemia, NO homeostasis is disturbed due to Ca<sup>2+</sup>-dependent e/nNOS activation. In endothelial cells, successive drop in NO concentration may occur due to both uncoupling of eNOS and/or successive inhibition of nNOS catalytic activity mediated by arachidonic acid-induced tyrosine phosphorylation of this enzyme. The reduced NO bioavailability triggers nuclear factor (NF)- $\kappa$ B activation followed by the induction of inducible NOS (iNOS) expression. In cardiomyocytes ischaemia also triggers the induction of iNOS expression during reperfusion. The massive amounts of NO which are subsequently produced following iNOS induction may exert on cardiomyocytes and the other cell types of cells of the heart, such as endothelial and smooth muscle cells, macrophages and neutrophils, op-

posing effects, either beneficial or toxic. The balance between these two double-faced actions may contribute to the final clinical outcomes, determining the degree of functional adaptation of the heart to ischaemia/reperfusion injury. In the light of this new vision on the critical role played by the cross-talk between n/eNOS and iNOS as well as the non enzymatic NO production by nitrite, we have reason to believe that new pharmacological measurements or experimental interventions, such as ischaemic preconditioning, aimed at counteracting the drop in NO levels beyond the normal range of NO homeostasis during early reperfusion can represent an efficient strategy to reduce the extent of functional impairment and cardiac damage in the heart exposed to ischaemia/reperfusion injury.

## Keywords

Cardiology, ischaemic heart disease, nitric oxide/NO

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## Introduction

In most cell types and tissues constitutively expressed nitric oxide synthases (NOS), neuronal NOS (nNOS) and endothelial NOS (eNOS), guarantee the production of “physiological” or “tonic” amounts of nitric oxide (NO) (presumably < 50 nM) (1, 2). NO thereby plays a critical role in a number of fundamental events, such as vascular tone regulation, retrograde neurotransmission, long-terminal potentiation and immune response as well as venous thromboembolism and ischaemic cardiovascular events (3–6). “Physiological” amounts of NO, however, continuously fluctuate according not only to the change in the concentration of

enzyme substrates, L-arginine and oxygen, enzyme co-factors such as NADPH, FAD, FMN, tetrahydrobiopterine and Ca<sup>2+</sup>/calmodulin, but also to the change in serine- or tyrosine-phosphorylation state of the enzymes and protein-protein interactions (7–9). For example, release of neurotransmitters such as acetylcholine, serotonin and catecholamines temporally and locally induces rapid increase in NO production by enhancing n/eNOS activity in the cardiovascular system. Physical insults, such as shear stress, also contributes to the transitory enhanced production of eNOS-derived NO in the blood vessel (10, 11). Nevertheless, under physiological conditions, the “tonic” yield of NO may not exceed either lower or upper limits, thus maintaining the so-called “NO homeostasis”.

## Cross-talk between neuronal/endothelial NOS and inducible NOS under inflammatory conditions

Under pathophysiological conditions, such as inflammation, another isoform of NOS, inducible NOS (iNOS) becomes preeminent. Pro-inflammatory cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin 1- $\beta$  (IL1- $\beta$ ), together with other molecules, such as lipopolysaccharides (LPS) (3), locally and temporally induce the expression of iNOS not only in immune cells, but also in any other cells activated by these inflammatory molecules through their specific receptors. Time-spatially modulated overproduction of NO (in  $\mu\text{M}$  order) (2) by iNOS during its relatively brief period of expression (usually, up to a few days) is normally expected to exert beneficial effects, since invading microorganisms can quickly be eliminated by the cytotoxic action of massively produced NO. However, under circumstances in which, by any reason, expression of iNOS escapes its fine mechanisms of regulation, leading to time-spatially deregulated production of NO, excessive amounts of NO, by reacting with superoxide ( $\text{O}_2^-$ ), can trigger the production of highly toxic peroxynitrites, thereby inducing severe inflammatory damages to tissues and organs ultimately culminating in ulcer formation (3).

Until a decade ago, a general view on the role played by n/eNOS and iNOS was that they have a distinct role under physiological and/or pathological situations, ignoring any possible functional correlation between these enzymes. Starting from 1995, Mariotto et al. (11–13) and Colasanti et al. (14) reported a series of data indicating that during the early phase of inflammatory response n/eNOS and iNOS may functionally cross-talk at least in some *in vitro* cell culture systems, such as astrocytes and endothelial cells treated with LPS and proinflammatory cytokines (15, 16). The crucial point of this view, postulating that the beginning of inflammation is characterised by a rapid drop in NO concentrations beneath the putative lower limit of NO homeostasis, seems to be widely accepted. Evidence from an increasing amount of literature concerning this fall in NO concentrations exists for a number of cells and tissues (8, 17, 18). Since NO, at low physiological concentrations (presumably  $<50\text{ nM}$ ) (2), is a powerful suppressor of nuclear factor (NF)- $\kappa\text{B}$  activation (19), the situation in which NO concentrations drop drastically may favor the local activation of NF- $\kappa\text{B}$  and successive induction of the expression of NF- $\kappa\text{B}$ -dependent inflammatory genes, including iNOS. In accordance with this vision, the possible involvement of arachidonic acid (AA) in the inhibition of nNOS activity at the early phase of the inflammatory response has recently been proposed (20, 21). Any treatment counteracting the drop in NO at inflammatory sites may therefore represent a new strategy to prevent or treat tissue damages induced by a deregulated or exaggerated inflammatory process.

## Mutual cross-talk between neuronal/endothelial NOS and inducible NOS in heart I/R injury

NO homeostasis is deeply involved in the correct function of the heart (22), where all three isoforms of NOS are widely distributed (23). Under normal conditions eNOS is known to be expressed within the heart in the endothelium both of the endocardium and of the coronary vasculature including capillary and venular endothelium (24, 25), in cardiomyocytes (26), specialised cardiac conduction tissue (27), as well as in some human blood cells including monocytes, platelets and recently detected in red blood cells (28–30).

The isoform nNOS present in intrinsic neurons within the heart has been detected by selective staining with nNOS antibodies in the atria, along epicardial coronary arteries and in specialised cardiac conduction tissue such as the sinoarterial and arterioventricular nodes (31–39).

Cytokine- and LPS-inducible  $\text{Ca}^{2+}$ -independent iNOS has been detected *in vivo* and *in vitro* in cardiac cells including ventricular myocytes (40, 41), microvascular endothelial cells (42), fibroblasts (43, 44), vascular smooth muscle cells (45) and infiltrating inflammatory cells (46).

The role played by NO in myocardial ischaemia/reperfusion (I/R) injury remains confusing, principally due to the lack of careful characterisation of NOS isoforms involved in I/R injury in different animal species (47). The general view has been that I/R can induce iNOS expression and the resulting high concentrations of NO exert deleterious actions leading to cardiac injury. In this context, Jones and Bolli (48), after careful reanalysis of numerous evidence from literature, recently proposed that iNOS, when expressed in cardiac myocytes, is a protective rather than toxic enzyme. Furthermore, a recent review, elegantly describing the possibility to consider NO homeostasis as a target for drug additives to cardioplegia, pointed out the critical role played by eNOS during I/R (49, 50). This is in line with a current view that either exogenously administered or endogenously produced NO should be a common mediator in the protection of the heart against I/R injury (51). In the present review, the authors include a new angle of vision underlying the importance of the putative functional cross-talk between n/eNOS and iNOS occurring during I/R injury in the heart. Special attention is given to the putative different roles played by iNOS and n/eNOS in cardiomyocytes and other type of cells present in the heart, especially endothelial cells.

One of the hallmarks at the very early phase of cardiac ischaemia, as in inflammatory response, is the increased  $\text{Ca}^{2+}$  uptake in cardiomyocytes and endothelial cells, which may enable two possible downstream signaling pathways: i) activation of  $\text{Ca}^{2+}$ -dependent nNOS and/or eNOS, leading to a rapid and short-lived increase in the concentration of NO (3) and ii) activation of a soluble phospholipase A2 (sPLA2), leading to the production of AA, which is involved in the inflammatory response (19–21). Brief activation of n/eNOS is followed by rapid consumption of not only their substrate, L-arginine, but also their co-factors, thus triggering either

inhibition of enzyme catalytic activity or eNOS-catalysed production of  $O_2^-$  and other deleterious oxygen radicals (eNOS uncoupling) (49, 50, 52). Early observations on AA-related production of  $O_2^-$  (53, 54) may reflect this pathway. This is also in line with another report showing a toxic effect of AA in I/R injury in the heart (55). Recently it has also been shown that hypoxia/reoxygenation of isolated rat heart mitochondria causes cytochrom c release, oxidative modification of mitochondrial lipids and proteins and inactivation of certain enzymes susceptible to inactivation by peroxynitrite. Mitochondria respond to hypoxia/reoxygenation by increase of intramitochondrial ionised calcium and thereby stimulate mitochondrial nitric oxide synthase (mtNOS). The consequence is elevated peroxynitrite formation (oxidative stress) upon reoxygenation (56). In this context it is interesting to note that a neuronal NOS has been identified in isolated cardiac mitochondria. The similarity of mtNOS to the neuronal isoform was deduced by the absence of NO production in mitochondria of knockout mice for the neuronal, but not the endothelial or inducible, isoforms (57).

According to recent reports by Cantoni's group (20, 21), AA enhances the catalytic activity of tyrosine kinase which phosphorylates nNOS in PC12 (rat pheochromocytoma), NIE-115 (mouse neuroblastoma) and C6 (rat glioma) cells, leading to rapid loss of the enzymatic activity. If a similar event happens also in the ischaemic heart, an initial  $Ca^{2+}$ -dependent short-lived increase in NO production by n/eNOS should be followed by a consistent decrease in NO concentration due to both eNOS uncoupling (lack of substrate and co-factors) (49, 50) and inhibition of the catalytic activity of nNOS by tyrosine-phosphorylation of the enzyme (15). Data on the dramatic decrease in NO production or bioavailability especially at the onset of reperfusion has been recently reported. Furthermore, uncoupling of eNOS can be prevented by arginine or NO supplementation with certain NO-donors (1, 50, 52), thus suggesting the importance of the presence of "tonic" amounts of NO at the onset of reperfusion. The proposed trigger of low NO concentrations (cross-talk) has been shown by *in vivo* NO measurements with a porphyrinic microsensor (55, 58) to be evident at least at the onset of reperfusion after a certain time of ischaemia (1, 50). In addition, an elevation in the concentrations of AA has also been reported in the reperfused heart (54, 59), as well as the prevention of induction of iNOS by the novel NO-Donor S-Nitroso human serum albumin given after LPS challenge in a rat model of endotoxaemia (60). Another mechanism which seems to occur is the up-regulation of L-arginase during I/R and this enzyme may compete with NOS for arginine (61). The enzyme arginase metabolises arginine to ornithine and urea. Indeed it has been shown that inhibition of arginase with the specific arginase inhibitor N-omega-hydroxy-nor-L-arginine mediates cardioprotection during I/R. The inhibition of arginase activity increases the bioavailability of NO by shifting utilisation of the substrate arginine from arginase towards NOS (62).

A recent report has documented that the drop in NO concentration occurring during cardiac I/R injury may play a prominent role in triggering NF- $\kappa$ B activation and/or successive induction of iNOS expression during the reperfusion phase. In eNOS knock-

out (KO) mice, I/R induced a superinduction of iNOS, due, at least in part, to enhancement of NF- $\kappa$ B activation, which was triggered by the very low levels of NO in these animals (63, 64). In agreement with these results an increased activity of NF- $\kappa$ B was also observed in rats receiving prolonged administration of the NOS inhibitor L-NAME (NG-nitro-L-arginine methyl ester) (65).

As already mentioned, the role played by iNOS in the I/R-induced heart damage remains controversial. Studies have shown both beneficial (66–71) and detrimental effects of iNOS (72–75). More recently, convincing findings by Bolli et al. documented that induction of iNOS in cardiac cells exposed to I/R injury is preeminent in providing cardioprotection (47, 51).

In considering the relevant involvement of NO in I/R-elicited cardiac injury, the role played by endothelial, smooth muscle or immune cells should not be underscored. In contrast to the acclaimed, putative protective role of iNOS in cardiomyocytes, deregulated iNOS expression in cardiac endothelial cells and other cell types, resulting in overabundant production of NO, might further extend the original damage caused by the ischaemic insult, by enhancing the toxic aspect of inflammation and/or increasing haemodynamic alterations due to massive vasodilatation.

The lipophilic nature of NO makes this situation more complex. A consistent part of NO produced in cardiomyocytes may reach neighbouring cells and amplify NO-mediated signalling response by means of a paracrine mechanism of action. Whereas NO per se is not harmful, some of the reaction products (hydroxyl radical, nitroxyl radical and nitrosonium cation) resulting from high peroxynitrite formation in the cell (e.g. after eNOS/iNOS uncoupling and/or reaction of NO with  $O_2^-$  produced by NAD(P)H oxidase) are highly cytotoxic substances. Thus, the excessive amount of NO produced in cardiomyocytes and other cell types during the reperfusion phase following iNOS induction may contribute to further the extent of I/R injury in the heart. It may be possible that iNOS induction is sustained, at least in part, by the inflammatory process occurring in endothelial and immunocompetent cells within the ischaemic area of the heart.

Furthermore, the description that either endogenously produced or exogenously administered NO represents one of the most important defences against myocardial I/R injury (48) provides a new connotation. In this regard, any increase in NO production achieved by different means (e.g. arginine or NO supplementation with NO donors, angiotensin converting enzyme inhibitors, specific endothelin receptor antagonists, calcium antagonists etc.) (49) within the ischaemic myocardium, as long as moderate, may be beneficial to the heart by preventing the drop in NO concentration at the onset of reperfusion and NF- $\kappa$ B activation. Indeed, an increase in NO bioavailability may lead to the down regulation of iNOS expression (16) or even prevent iNOS induction (60), thereby limiting or preventing the toxic consequences of the inflammatory response.

In addition, translocation of nNOS may play a role. The report by Sun et al. (76) shows that in isoproterenol treated ischaemic/reperfused mice hearts nNOS can translocate from the sarcoplasmic reticulum to the sarcolemma with significantly more nNOS translocation to caveolin-3 and more eNOS associated with car-

diomyocyte caveolin-3 in females. These data also indicate that eNOS and nNOS both play roles in the gender differences observed in I/R-injury under adrenergic stimulation. Furthermore, females have been shown to be more protected than males from I/R injury. This is either due to higher production of eNOS-derived NO in mice with cardiac overexpression of  $\beta_2$ -adrenergic receptors (77) and/or increased nNOS translocation and higher S-nitrosylation of L-type  $\text{Ca}^{2+}$  channels (76).

The cardioprotective effects afforded by the early and late ischaemic preconditioning (PC) (78, 79) deserve further attention. Ischaemic PC, originally described as an immediate adaptation of the heart to brief coronary occlusion, was subsequently found to be a biphasic phenomenon with the early phase occurring immediately after ischaemic PC stimulus and lasting 2–3 hours (h) and a late phase that becomes apparent after 12–24 h and lasts 3–4 days (79). Endogenous NOS-derived NO is not a trigger or mediator of the early phase of ischaemic PC against infarction either in rabbits (80) or pigs (81). However, NOS-derived NO plays a fundamental role for initiating and mediating the delayed phase of ischaemic PC's protection (47). In the late phase, NO produced by eNOS and to some extent by iNOS due to partial induction of the enzyme (48, 82–85) result in moderately enhanced NO bioavailability. Endogenous NO is here a key trigger and mediator of the late phase of ischaemic PC (86), and the late phase of protection clearly results from altered expression of protective proteins (for review, see [87]).

### Non enzymatic NO production – Cardioprotective effect of nitrite against I/R injury

Beside NO produced by the NOS isoforms NO can also result from nitrite, once thought to be a physiologically inert oxidation product of NO. Nitrite has now been recognised to be an endocrine storage form of NO (88, 89). Whereas plasma nitrite concentrations are in the nanomolar range (approximately 100 nM) cardiac tissue contains higher concentrations (1–10  $\mu\text{M}$ ) under basal conditions (90). In conditions of low oxygen and pH, nitrite is reduced, via a reaction that is catalysed by various heme containing proteins, to bioavailable NO. Thus, nitrite acts as an NO “pro drug” that can be selectively reduced during hypoxic or ischaemic conditions to generate biologically active NO and therefore exerts a number of NO-like actions (91). As already described, the bioavailability of NOS-derived NO is scarce during the early phase of I/R. However, an increasing body of evidence indicates that NO produced by proteins with nitrite reductase activity such as xanthine oxidoreductase and deoxygenated myoglobin (92, 93) exert a cardioprotective action after I/R. In addition, intravenous nitrite therapy has by a number of investigators been shown to protect against myocardial I/R-injury in different animal models (94–97). The cytoprotection afforded by nitrite bears many parallels to the afore-mentioned ischaemic PC in the heart (91). Mechanistically, it is known that like nitrite, ischaemic PC attenuates mitochondrial

ROS generation and consequently oxidative damage to tissue. It has also been shown that both delayed and classical PC increased nitrite tissue concentrations (51). These data support the importance of NOS independent sources of NO such as nitrite in ischaemic PC. The mechanism of the cytoprotection by nitrite seems to occur at the level of the mitochondria. Both NO and nitrite-derived NO have been shown to inhibit complex I by mediating the reversible post-translational S-nitrosation of the enzyme (91). This S-nitrosation of complex I leads to an attenuation of ROS generation and prevents cytochrom c release from the mitochondria upon reoxygenation due to reperfusion (95). These notions further strengthen the critical role of NO bioavailability at the early and late phase of I/R.

### Clinical relevance of iNOS expression in the heart

In the clinical setting, whenever myocardial ischaemia ensues following acute coronary occlusion or vasospasm, the main therapeutic goals are the protection of the ischaemic myocardium from necrotic and apoptotic cell death and the prevention of the post-ischaemic endothelial dysfunction, which can result in the so-called no-reflow phenomenon. Similar considerations may also apply to the mild forms of iatrogenic I/R injury given to the human heart during cardioplegic arrest and subsequent reperfusion, as well as during balloon inflation in the course of percutaneous transluminal coronary angioplasties (PTCA). Consequently, interventions aimed at reversing the impairment of NO bioavailability occurring in the most precocious stages of reperfusion may contribute to counteract and possibly downsize post-ischaemic endothelial dysfunction and impairment of cardiac contractility. Such interventions should be efficacious even if administered after ischaemia, as long as initiated at the very incipit of coronary revascularisation. Likewise, complementary pharmacological agents capable of preventing the late iNOS-derived NO overproduction, with subsequent peroxynitrite formation, may be in turn clinically advantageous.

### Conclusive remark

NO homeostasis maintained by n/eNOS contributes to regulate cardiac function under physiological conditions (2). At the early stages of ischaemia, consequently to the abrupt increase in  $\text{Ca}^{2+}$  influx, NO homeostasis is disturbed due to e/nNOS activation. In endothelial cells, after the initial e/nNOS activation the drop in NO concentration may occur due to both uncoupling of eNOS and/or successive inhibition of nNOS catalytic activity mediated by AA-induced tyrosine phosphorylation of this enzyme. In this context the role and timely contribution of NOS-independent sources of NO such as nitrite have to be taken into account. The reduced NO bioavailability triggers NF- $\kappa\text{B}$  activation followed by iNOS expression. In cardiomyocytes ischaemia also triggers the induction

of iNOS expression during reperfusion. However, further investigations are needed in order to elucidate whether the drop in NO concentration observed in cardiac myocytes occurs between the initial increase in eNOS activity and the induction of iNOS expression. The massive amounts of NO which are subsequently produced following iNOS induction may exert on cardiomyocytes and the other cell types of cells of the heart, such as endothelial and smooth muscle cells, macrophages and neutrophils, opposing effects, either beneficial or toxic. The balance between these two double-faced actions may contribute to the final clinical outcomes, determining the degree of functional adaptation of the heart to I/R injury. In the light of this new vision on the critical role played by the cross-talk between n/eNOS and iNOS, we have reason to believe that new pharmacological measurements or experimental interventions, such as ischaemic PC, aimed at counteracting the drop in NO levels beyond the normal range of NO homeostasis during early reperfusion can represent an efficient strategy to reduce the extent of functional impairment and cardiac damage in the heart exposed to I/R injury.

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