

Nitric oxide synthase in post-ischaemic remodelling: new pathways and mechanisms

Boris Manoury[†], Virginie Montiel, and Jean-Luc Balligand*

Pole of Pharmacology and Therapeutics (FATH), Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCL), 52 avenue Mounier, 1200 Brussels, Belgium

Received 10 October 2011; revised 2 December 2011; accepted 27 December 2011; online publish-ahead-of-print 6 January 2012

Abstract

The three isoforms of nitric oxide synthase (NOS), spatially confined in specific intracellular compartments in cardiac cells, have distinct roles in the regulation of contractility in pathophysiological situations. Recently, evidence has emerged that implicates NOS in modulating myocardial remodelling during cardiac stress, including after ischaemic insults. As long as they remain in a coupled state the NOS mostly attenuate hypertrophic remodelling through both cGMP-dependent and independent mechanisms. We review the evidence provided from the phenotype of genetic mouse models as well as from *in vitro* cell experiments dissecting the signalling effectors involved in the NOS-mediated regulation that justify new therapeutic interventions on the NOS–cGMP axis to attenuate the development of heart failure.

Keywords

Nitric oxide synthase • Protein kinase G • Cardiac remodelling • Hypertrophy • Heart failure

This article is part of the Spotlight Issue on: Reducing the Impact of Myocardial Ischaemia/Reperfusion Injury

This review summarizes the evidence that contributed during the last decade to shedding light on the role of the three nitric oxide synthase (NOS) isoforms in orchestrating post-ischaemic cardiac remodelling. Data obtained from non-ischaemic models of cardiac remodelling as well as ischaemia/reperfusion (I/R) models are discussed in order to broaden the view of mechanisms involving NOS isoforms.

1. Recap on NOS distribution and function in cardiovascular tissues

Nitric oxide (NO) is a key universal signalling molecule in the cardiovascular system, acting on both acute and more chronic pathways. Nitric oxide is produced from L-arginine by three NO synthases coded by their respective gene, namely *NOS1* for neuronal NOS (nNOS), *NOS2* for inducible NOS (iNOS), and *NOS3* for endothelial NOS (eNOS), expressed in various cardiovascular cell types.¹ Nitric oxide may therefore influence cardiac function through both paracrine and autocrine signalling. In addition, differential subcellular localization of NOS isoforms supports distinct effects on excitation–contraction (E–C) coupling in cardiomyocytes. For instance, nNOS is co-immunoprecipitated with the cardiac ryanodine receptor 2 in

sarcoplasmic reticulum (SR) membranes of normal rabbit and human hearts,² suggesting a modulation by nNOS-derived NO of SR calcium release. Previous evidence also suggested the presence of NOS in mitochondria.³ In cardiomyocytes, a subset of eNOS is enriched in plasmalemmal and T-tubular caveolae, where it is associated with caveolin-3, the myocyte-specific structural protein of caveolae,¹ pointing to a role in modulation of membrane-initiated signalling. In addition, such subcellular localization may be dynamic and depends on pathophysiological conditions; accordingly, nNOS can be redistributed from SR membranes to the sarcolemma of cardiomyocytes with the development of heart failure (HF).⁴

Both nNOS and eNOS are constitutively expressed in the heart but produce small amounts of NO, tightly regulated by post-transcriptional mechanisms (best characterized for eNOS), involving the following mechanisms: (i) interaction with $[Ca^{2+}]$ –calmodulin; (ii) phosphorylation (e.g. through protein kinase B on Ser-1177 for human eNOS) with increased activity; (iii) dephosphorylation (e.g. by protein phosphatase 2A/calcineurin on Thr-495 for human eNOS, allowing full enzyme activation; (iv) association of reduced tetrahydrobiopterin (BH₄) that, together with the substrate L-arginine, stabilizes the assembly of two NOS subunits as a functional dimer and sustains catalytic NO production; conversely, monomeric NOS functions as a

[†] Present address. Boris Manoury, Signalisation et Physiopathologie Cardiaque, Inserm UMR-S 769 – LabEx LERMIT, Faculté de Pharmacie – IFR141, 5 rue JB Clément, 92296 Châtenay-Malabry Cedex, France. Email: boris.manoury@u-psud.fr

* Corresponding author. Tel: +32 2 764 52 60, Email: j.l.balligand@uclouvain.be

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superoxide-producing enzyme, and a displaced equilibrium between these two functional states ('uncoupling' of the NOS) promotes the reaction of superoxide with NO to form peroxynitrite (ONOO⁻), further increasing oxidation of BH₄, NOS uncoupling, and oxidative/nitrosative stress; and (v) chaperone proteins, such as heat shock protein 90, that promotes a sustained activation of the enzyme.¹ In contrast, iNOS produces larger amounts of NO and is mainly modulated by transcriptional regulation; its expression can be induced in a wide range of cell types upon pro-inflammatory stimulation.

Besides classically promoting vasodilatation and angiogenesis, and opposing platelet adhesion, vascular smooth muscle cell proliferation, and migration, NO has direct and indirect pleiotropic influences on cardiac function, e.g. optimization of E–C coupling or modulation of mitochondrial respiration. More recently, the ability of NOS to modulate redox signalling by balancing the production and reactivity of reactive oxygen species has come into focus.^{1,5–7}

Among downstream effectors, NO activates soluble guanylyl cyclase (sGC), leading to the production of cGMP and subsequent activation of cGMP-dependent protein kinase G type 1 (PKG1). PKG1 exerts negative inotropic effects by diminishing L-type Ca²⁺ channel currents and through desensitization of cardiac myofilaments to Ca²⁺ following troponin I phosphorylation (recently reviewed in reference⁸). Specific pools of cGMP also regulate the activity of isoforms of the phosphodiesterases (PDE) family that hydrolyse cyclic nucleotides, including cAMP and cGMP itself. Phosphodiesterases probably play an important role in compartmentation of cyclic nucleotides pools. Nitric oxide also exerts biological effects through cGMP-independent mechanisms, including S-nitrosylation of key target proteins, as developed below (section 3.2; Figures 1 and 2).

2. Which NOS isoform in post-ischaemic remodelling? Evidence from *in vivo* transgenic models

Following ischaemia, the myocardium undergoes repair processes to heal the ischaemic zone and remodels the remaining tissue in order to cope with the haemodynamic stress resulting from the loss of functional muscle.⁹ We will now review the evidence implicating NOS and NO in myocardial remodelling, as deduced from the phenotype of mouse models with genetically engineered NOS submitted to various cardiac stresses [e.g. by transaortic constriction, myocardial infarction (MI), followed or not by reperfusion (ischaemia/reperfusion; I/R) *in vivo* or *ex vivo*; see also Table 1].

2.1 Is nNOS protective against ischaemic injury and adverse remodelling?

The course and degree of remodelling is likely to be influenced by the regulation of E–C coupling and its modulation by the NOS in the remaining, viable myocardium. The way in which nNOS regulates E–C coupling in the healthy heart is complex, as suggested by recent (and sometimes contradictory) data using genetic models. One group found that deletion of the *NOS1* gene in mice enhanced contractility,^{10,11} with increased Ca²⁺ transients and cell shortening at baseline, whereas this was not observed by others.¹² During β -adrenergic stimulation, however, the former group found that myocytes from *NOS1*^{-/-} mice exhibited an increased contractile

response,¹⁰ whereas another group observed depressed contractility.¹³ Nevertheless, a common feature of all *NOS1*^{-/-} strains studied is that lusitropic parameters are depressed compared with wild-type.^{11,14} Several mouse strains with cardiac overexpression of nNOS (*NOS1*_{tg}) were also generated but, depending on their origin, their phenotype either suggested that nNOS has a negative effect on L-type Ca²⁺ channel current density, Ca²⁺ transients, and cell shortening (*NOS1*_{tg} generated by Burkard *et al.*)¹⁵ or, on the contrary, a positive influence on these inotropic parameters (*NOS1*_{tg} generated by Loyer *et al.*)¹⁶ As for relaxation, Loyer *et al.* consistently reported an accelerated decay of Ca²⁺ transient and relaxation time together with increased phosphorylation of phospholamban at serine 16 and threonine 17,¹⁶ but Burkard *et al.*¹⁵ observed prolonged relaxation time and reduction of phospholamban Ser-16 phosphorylation. Although the causes of these contradictory results are unclear, the background of the strain used for the generation of transgenic mice and also the animals used as experimental controls (e.g. littermate controls vs. age-matched C57BL/6 mice) may be of importance. Also, the differential subcellular localization of endogenous vs. recombinant nNOS (e.g. in Burkard *et al.*)¹⁵ may explain some of the variability between studies. Moreover, even though separate roles and compartmentation of NOS isoforms have clearly emerged, some redundancy may exist, and the consequence of permanent deletion of one isoform may be masked by compensation from the others. Of note, the phenotype of systemic *NOS1*^{-/-} animals *in vivo* may be confounded by an imbalance in parasympathetic stimulation, because NO produced by nNOS in neurons produces presynaptic facilitation of cardiac vagal control and therefore counteracts sympathetic regulation.⁵ Finally, interaction of nNOS with alternative effectors of Ca²⁺ handling, such as the sarcolemmal calcium pump (PMCA4b), may also participate in nNOS-mediated modulation of β -adrenergic stimulation.¹⁷

As expected, despite the large number of studies performed during the last decade, the exact role played by nNOS in the response to cardiac stress and subsequent remodelling is still being debated. An increased expression and activity of nNOS has been reported after MI-induced HF in rats, coupled with the translocation of this enzyme to the sarcolemma through interactions with caveolin-3.⁴ Here pharmacological inhibition of nNOS (with N5(1-imino-3-butenyl)-L-ornithine; 0.1 μ M) improved cardiac contractility in failing hearts. Increased expression and activity were also observed in end-stage congestive HF in patients with idiopathic dilated cardiomyopathy, whereas eNOS expression was lower than in control patients.¹⁸ This raises the question of whether this nNOS up-regulation is adaptive or deleterious.

Early work had shown that systemic deletion of *NOS1* in mice led to spontaneous concentric left ventricle (LV) remodelling,¹³ and this was exacerbated following MI.^{19,20} Post-MI, *NOS1*^{-/-} mice showed a faster and more severe LV dilatation compared with their wild-type controls. They responded to dobutamine with a dramatic fall in LV contractility.¹⁹ Another group showed greater mortality, worse left ventricular fractional shortening, dilatation with a higher LV diastolic diameter, and increased superoxide production after MI in *NOS1*^{-/-} mice.²⁰ Despite the caveat on the interpretation of the phenotype of systemic knockout animals, these data would support a protective role for cardiac nNOS activity after MI, although it potentially contributes to LV dysfunction through β -adrenergic hypo-responsiveness.

More recent data using tissue-specific overexpression models provided further evidence that nNOS can limit functional alterations

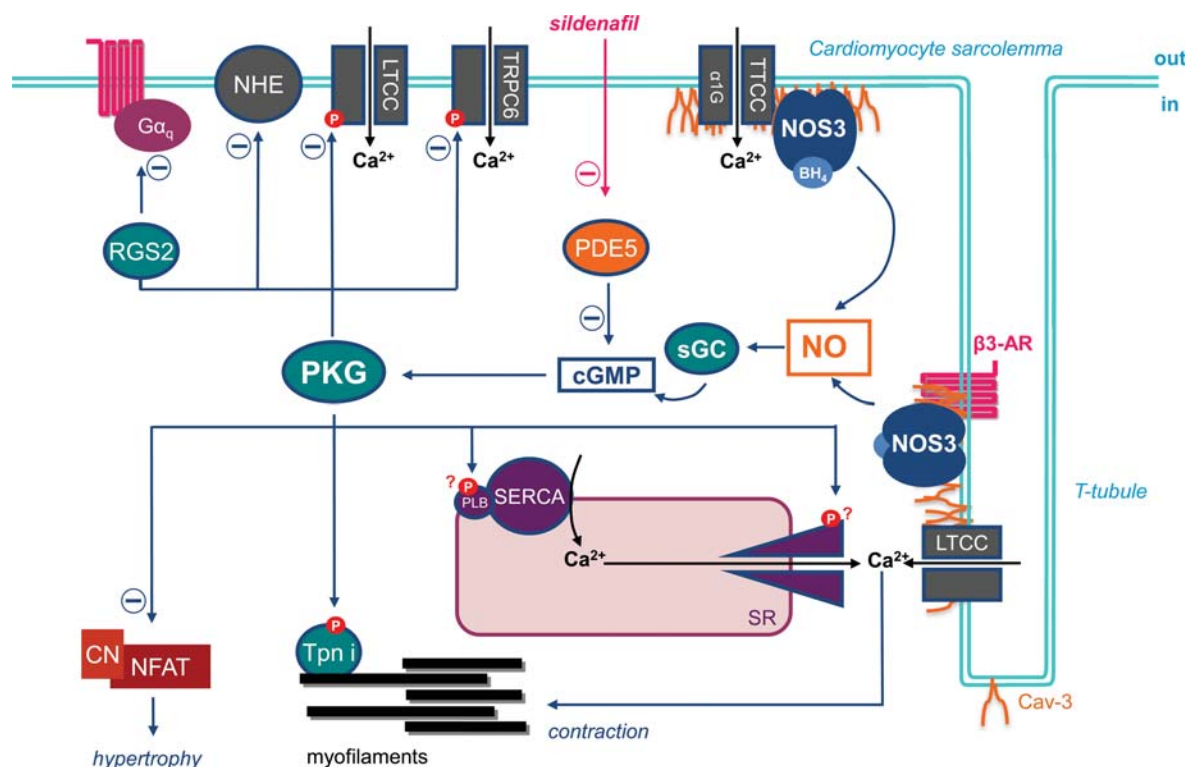


Figure 1 Cyclic GMP-mediated mechanisms for nitric oxide synthase (NOS) regulation of excitation–contraction coupling and hypertrophy in cardiac myocytes. Endothelial NOS (coded by *NOS3*) localized to the sarcolemma modulates the function of several effectors of excitation–contraction coupling and hypertrophy through activation of the soluble isoform of guanylyl cyclase (sGC) and cGMP-dependent protein kinase (PKG). The latter modulates the excitation–contraction coupling through changes in phosphorylation of the L-type calcium channel (LTCC), myofilament-associated troponin I (Tpn i) and perhaps, phospholamban (PLB) and the ryanodine receptor. Endothelial NOS also associates with the $\alpha 1G$ subunit of the T-type calcium channel (TTCC) in a subset of rafts/caveolae, where it is probably activated by calcium entry through this channel; similar downstream cGMP/PKG activation then attenuates pro-hypertrophic pathways, such as calcineurin (CN)/nuclear factor of activated T cells (NFAT); in addition, PKG was shown to inhibit hypertrophy through regulator of G-protein signalling-2 modulation of $G\alpha_q$ signalling, inhibition of transient receptor potential cation channel, subfamily C, member 6 (TRPC6) and the Na^+-H^+ exchanger, NHE. Sildenafil-mediated inhibition of phosphodiesterase (PDE)-5 amplifies specific pools of cGMP, thereby potentiating some PKG downstream effects, e.g. on troponin I; similar pools may be activated by β_3 -adrenoceptor (β_3 -AR) stimulation of endothelial NOS to modulate hypertrophy (see main text for details and references). Cyclic GMP may also target cAMP catabolism through other isoforms of phosphodiesterases (e.g. PDE-2 and PDE-3) and thereby modulate the contractile response to catecholamines (not shown). Abbreviations: BH₄, tetrahydrobiopterin; Cav-3, caveolin-3; SR, sarcoplasmic reticulum.

subsequent to pressure-overload or post-ischaemic damage. In a transaortic constriction (TAC)-induced pressure-overload model, mice with cardiomyocyte-restricted nNOS overexpression showed concentric hypertrophy with preserved LV fractional shortening, whereas control mice displayed eccentric remodelling and loss of function. As mentioned above (*NOS1_{tg}* used by Loyer et al.),¹⁶ this was associated with greater L-type Ca^{2+} channel current, Ca^{2+} transients and sarcoplasmic reticulum Ca^{2+} load in transgenic mice compared with wild-type. This suggests that nNOS may limit the transition to adverse remodelling and HF through preservation of calcium cycling.¹⁶

Some of the protective effects of nNOS could derive from its capacity to counteract the nitroso-redox imbalance that occurs after ischaemia by inhibiting xanthine oxidoreductase (XOR) and mitochondrial reactive oxygen species production.⁶ Indeed, cardiac overexpression of nNOS confers protection from I/R injury, in part through inhibition of XOR activity. In these conditions, nNOS was also shown to be translocated to mitochondria via a heat shock protein 90-dependent mechanism. In addition to XOR inhibition,

overexpression of nNOS was associated with increased mitochondrial nitrite levels, dithiothreitol-sensitive inhibition of cytochrome c oxidase activity, and lowered ATP consumption.²¹ Whether such regulation observed in an overexpression system fully applies to nNOS at endogenous expression levels remains an open question. Nevertheless, the conjunction of nNOS effects to inhibit XOR and mitochondrial ROS production, as well as perhaps L-type Ca^{2+} channel current, each supported by the enzyme's subcellular localization in specific settings (I/R or HF) was proposed to contribute to protective effects of the enzyme against post-stress remodelling.

2.2 iNOS

At first glance, the numerous studies on stress-induced cardiac remodelling using *NOS2* transgenic animals (summarized in the Table 1) would not allow univocal conclusions to be drawn on the role of this isoform, but some apparent contradictions may be explained by model-specific features. In one study, cardiac-specific overexpression of iNOS in mice was reported to induce all the features of severe adverse remodelling with conduction defects, and

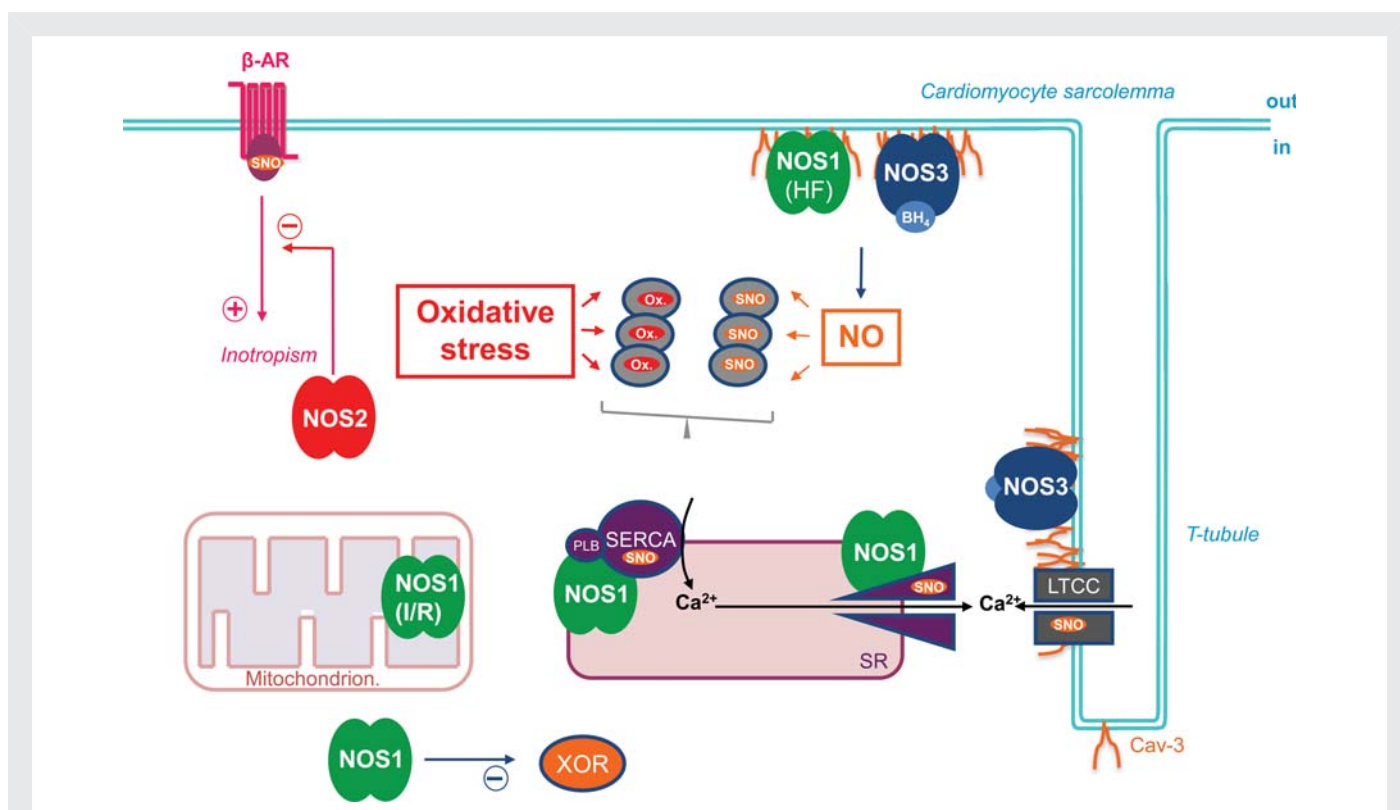


Figure 2 Cyclic GMP-independent mechanisms for NOS regulation of oxidant stress and excitation–contraction coupling. Spatially confined NOS may modulate the cardiac myocyte response to mechanical and ischaemic stress via S-nitrosylation (SNO) of several protein effectors of excitation–contraction coupling. In addition to direct modulation of the function of these effectors, one hypothesis proposes that S-nitrosylation is balanced with oxidative stress and may shield proteins against irreversible oxidative damage. Neuronal NOS (coded by *NOS1*) was also shown to protect from oxidative damage through inhibition of xanthine oxidoreductase (XOR); similar antioxidant mechanisms may be operative after translocation of neuronal NOS to mitochondria. In the setting of ischaemia/reperfusion (I/R), inducible NOS (coded by *NOS2*) may also exert cardioprotective effects through protein kinase G (PKG) targeting of the mitochondrial K_{ATP} channel (not shown). In other settings, however, inducible NOS exerts a negative influence on β -adrenergic inotropism, although the mechanisms remain unclear (see main text for details and references). Abbreviations: BH_4 , tetrahydrobiopterin; HF, heart failure; SR, sarcoplasmic reticulum.

increased mortality, associated with myocardial peroxynitrite production.²² In contrast, another iNOS-overexpressing mouse strain exhibited no cardiac dysfunction, and this apparent protection was attributed to buffering of excessive NO by myoglobin.^{23,24} As the milder phenotype cannot be attributed to lower NOS activity in the latter study, the divergence could be related to differences in the genetic construction of the two models (cardiac-specific non-conditional²³ vs. tetracycline-inducible transactivator, co-expressing LacZ²²).

In wild-type mice, iNOS expression and activity were shown to increase following myocardial infarction in the infarcted area²⁵ (approximately four- to five-fold increase of S-methylisothiourea sulfate-inhibitable activity, peaking at 72–96 h and back to normal at day 14 post-infarction²⁶), but not in the non-infarcted region. However, 5 months after MI, iNOS was no longer detected in the infarcted region but was expressed in the myocytes of remote regions of the myocardium.²⁷

Long-term administration of selective iNOS inhibitors to rats²⁸ or *NOS2* gene deletion in mice^{25,27} also improved cardiac function after MI, with significant reduction in mortality, suggesting that induction of iNOS post-MI exerts a deleterious influence on cardiac remodelling, although this was not observed in another study.²⁹ The

protection conferred by iNOS deletion appeared at late stages of remodelling (4 months) rather than determining infarct size or early LV remodelling during the first month after MI.²⁷ In pressure-overload models, deletion of *NOS2* did not prevent adverse remodelling,³⁰ whereas it delayed the establishment of cardiac dysfunction on the long term in another study.³¹ Therefore, some of variance in the observed phenotypes may be explained by the timing of the analysis or by different experimental conditions (e.g. extent of the inflammatory reaction).

Indeed, expression and activity of iNOS increases not only in cardiac myocytes in HF models (approximately two-fold increase)³² but also in other infiltrating inflammatory cells, as observed in transgenic mice with cardiac-specific overexpression of tumour necrosis factor- α .³³ Pharmacological inhibition of iNOS using ONO-1714 had no effect on basal contractility but improved β -adrenergic inotropic responsiveness in these mice.³³ Interestingly, other reports consistently associate the deletion²⁵ or the inhibition of iNOS with enhanced inotropic response to β -adrenergic stimulation in human³⁴ or rat³² failing hearts, as initially demonstrated in isolated rat cardiomyocytes.³⁵ Thus, iNOS may have a causal role in the hypo-responsiveness to β -adrenergic receptor stimulation that, in turn, may participate in the development of LV dysfunction.

Table 1 Overview of the cardiac phenotypes of mouse models with genetic deletion or overexpression of the three nitric oxide synthase (NOS) isoforms

	Reference	Model	Observations/conclusions
NOS1 (nNOS)	Barouch ¹⁰³	<i>NOS1</i> ^{-/-} , ageing	Age-dependent cardiac hypertrophy with concentric LV remodelling. Exacerbated age-dependent cardiac hypertrophy
	Barouch ¹⁰⁴	Combined <i>NOS1</i> ^{-/-} , <i>NOS3</i> ^{-/-}	Exacerbated pathological remodelling (LV dilatation); lower response to dobutamine
	Dawson ¹⁹	<i>NOS1</i> ^{-/-} , MI	Increased mortality; remodelling
	Saraiva ²⁰	<i>NOS1</i> ^{-/-} , MI	Increased XOR activity
	Khan ⁶⁵	<i>NOS1</i> ^{-/-}	Exacerbated XOR-mediated inhibition of cardiac excitation–contraction coupling
	Burkard ²¹	Cardiomyocyte-specific <i>NOS1</i> _{tg} , I/R	Myocardial protection in perfused hearts and <i>in vivo</i> ; decreased reactive oxygen species production via inhibition of XOR; increased nitrite levels and depressed cytochrome c oxidase activity in mitochondria
NOS2 (iNOS)	Loyer ¹⁶	Cardiomyocyte-specific <i>NOS1</i> _{tg} , TAC	LV hypertrophy, but less dilatation and eccentric remodelling; preserved Ca ²⁺ handling in isolated cardiomyocytes
	Burger ⁶⁹	MI, <i>NOS1</i> ^{-/-} vs. WT	Increased ventricular arrhythmia and mortality
	Sam ²⁷	MI, <i>NOS2</i> ^{-/-}	Improved LV contractile function; reduced apoptotic cell death
	Liu ¹⁰⁵	MI, <i>NOS2</i> ^{-/-}	Preserved cardiac function and improved survival in <i>NOS2</i> ^{-/-} vs. WT
	Jones ²⁹	Severe MI, <i>NOS2</i> ^{-/-}	No attenuation of severe congestive failure
	Mungrue ²²	Cardiomyocyte overexpression of NOS2	Peroxyntitrite (ONOO ⁻) generation, heart block and sudden death
	Heger ²³	Cardiomyocyte overexpression of NOS2	No severe cardiac dysfunction
	Bolli ¹⁰⁶	Myocardial stunning/late preconditioning	Protective effect of NOS2 in late phase of ischaemic preconditioning
	Das ¹⁰⁷	Sildenafil preconditioning on adult cardiomyocytes	NOS2-mediated protective effects of late preconditioning
	Marfella ¹⁰⁸	MI, <i>NOS2</i> ^{-/-} , induced diabetes in mice	Non-diabetic: injury more severe in <i>NOS2</i> ^{-/-} vs. WT. Diabetic: injury more severe in WT vs. <i>NOS2</i> ^{-/-}
	West ³⁹	Cardiac-specific expression of NOS2, I/R	Cardioprotection and decrease in reperfusion-induced oxygen radicals and inhibition of mitochondrial swelling and permeability transition
	Saito ²⁸	MI, selective iNOS inhibitor S-methylisothiourea long term	Improved cardiac function; decreased mortality, lung water content, infarct size, cardiomyocyte and LV hypertrophy
	Hataishi ³⁰	<i>NOS2</i> ^{-/-} , TAC	Similar LV hypertrophy in knockout vs. WT
	Gealekman ³²	Volume overload-induced heart failure in rats, NOS2 increased	β-Adrenergic hypo-responsiveness
	Dias ³¹	Pressure overload by aortic banding, <i>NOS2</i> ^{-/-} vs. WT	Increased basal cardiac function; improved response to β-adrenergic stimulation
	Kobayashi ¹⁰⁹	Dahl salt-sensitive hypertensive rats, selective NOS2 inhibition by aminoguanidine	Protection against cardiac dysfunction and vascular remodelling
NOS3 (eNOS)	Barouch ¹⁰³	<i>NOS3</i> ^{-/-}	Developed CHF by 5 months, probably subsequent to hypertension caused by vascular NOS3 deficiency
	Cappola ⁴²	<i>NOS3</i> ^{-/-}	25% increase in heart weight; 47 differentially expressed genes
	Ichinose ⁴³	<i>NOS3</i> ^{-/-} , TAC	Exacerbated CHF and LV dysfunction
	Ruetten ⁴⁴	<i>NOS3</i> ^{-/-} , chronic TAC	Concentric LV hypertrophy
	Jones ^{46,110}	Endothelium-specific <i>NOS3</i> _{tg} , MI and I/R injury	Attenuated MI-induced injury, but failed to protect significantly against post-ischaemic myocardial contractile dysfunction in mice
	Brunner ¹¹¹	Cardiomyocyte-specific <i>NOS3</i> _{tg} , I/R injury	Decreased infarct size; preserved LV function
	Scherrer-Crosbie ⁴⁵	<i>NOS3</i> ^{-/-} , MI	Worse systolic and diastolic basal function, decreased capillary density, increased myocyte hypertrophy and mortality at 4 weeks
	Janssens ⁴⁸	Cardiomyocyte-specific <i>NOS3</i> _{tg} , MI	Protection against MI-remodelling, not against fibrosis
	Szelid ¹¹²	Cardioselective <i>NOS3</i> gene transfer, I/R	Attenuated I/R injury; sustained reduction in myocardial infarct size and inflammatory cell infiltration
	Takimoto ¹¹³	TAC/PDE5A inhibition	Sildenafil prevented and reversed CHF
	Takimoto ¹¹⁴	TAC (severe), <i>NOS3</i> ^{-/-} vs. WT, +/- BH ₄	Increased oxydative stress, reversed by BH ₄ supplementation
	Moens ⁵⁰	TAC (4 weeks) +/- BH ₄ ; endothelium-specific BH ₄ production	Exogenous (but not endothelium-restricted) BH ₄ protected against remodelling by targeting principally myocytes and fibroblasts
	Buys ⁴⁷	Cardiomyocyte-specific NOS3 on <i>NOS3</i> ^{-/-} , TAC	Restoration of NOS3 protected against remodelling and cardiac dysfunction

Abbreviations: BH₄, tetrahydrobiopterin; CHF, chronic heart failure; I/R, ischaemia/reperfusion; LV, left ventricle/ventricular; MI, myocardial infarction; TAC, transaortic constriction; WT, wild-type; and XOR, xanthine oxidoreductase. *NOS1*_{tg}, *NOS1* transgenic mice; *NOS3*_{tg}, *NOS3* transgenic mice; PDE5A, Phosphodiesterase 5A.

The production of NO in all these conditions probably involves multiple cell types expressing iNOS within the myocardium,³³ as well as more than one NOS isoform. The cell- and isoform-specific contribution can hardly be resolved with systemic administration of NOS inhibitors or systemic *NOS2* genetic deletion. Isolated, single-myocyte contractility assays did establish the role of myocyte-restricted iNOS, as mentioned above,³⁵ but cell-specific genetic deletion would be needed to extend this role *in vivo*. Indirect evidence for a predominant role of myocyte iNOS was provided by a study using mice with cardiac-specific overexpression of calcineurin (CN/Tg mice) that also overexpress iNOS, produce tumour necrosis factor- α , and develop LV dysfunction. These mice were crossed into the *NOS2*^{-/-} background, and the resulting phenotype was compared with that of chimeric mice reconstituted with bone marrow from *NOS2*^{-/-} or wild-type mice in order to assess the specific role of iNOS in bone-marrow-derived infiltrating cells; this study pointed to iNOS in myocytes as the main culprit for the dysfunction.³⁶

Importantly, although the evidence points towards a deleterious effect of iNOS in post-ischaemic remodelling, this does not preclude short-term cardioprotective effects in the setting of I/R (for review, see reference³⁷). In a rat model of late ischaemic preconditioning (also called the second window of preconditioning), for instance, inhibition of iNOS at the time of the preconditioning stimulus did not abolish the delayed protection, but it did so if administered immediately before the ischaemic insult (index ischaemia), suggesting that iNOS may not be involved in the trigger stimulus, but is part of the delayed protection.³⁸ In fact, activation of cardiac NOS during ischaemic preconditioning may be biphasic, with eNOS activity peaking during the preconditioning stimulus and iNOS activity predominating 24 h later.³⁷ The mechanism of iNOS-mediated protection may involve a reduction of oxidant radicals that prevents mitochondrial permeability transition, as observed in cardiac myocyte-specific iNOS overexpressors submitted to I/R.³⁹

Therefore, both the intensity and the time window of iNOS overexpression may dictate its differential effect, either protective in the context of preconditioning or deleterious in the presence of sustained inflammatory stimuli, such as in late-stage cardiomyopathy.

2.3 eNOS

Endothelial NOS expressed in cardiac myocytes also regulates E–C coupling in physiological conditions. When overexpressed in cardiac myocytes, it attenuates the β_1 / β_2 -adrenergic increase in inotropy and chronotropy and reinforces the post-synaptic vagal control of cardiac contraction.⁴⁰ In addition, eNOS contributes to the fine tuning of contractility by mediating the slow increase in cardiac force in response to stretch (i.e. the Anrep effect).^{1,41}

As with nNOS, mice with systemic deletion of the *NOS3* gene developed hypertrophy with ageing, partly secondary to hypertension.¹³ They also developed more LV hypertrophy, fibrosis, and dysfunction following pressure overload in three studies,^{42–44} one of which included a group of *NOS3*^{-/-} mice treated with the anti-hypertensive drug hydralazine in order to reduce the confounding effects of hypertension.⁴³ Moreover, the induction of MI in systemic *NOS3*^{-/-} mice induced worse systolic and diastolic function, decreased capillary density, and mortality at 4 weeks.⁴⁵

Although these studies highlight a protective role for eNOS from remodelling, the use of systemic knockout mice does not resolve the question of whether the 'protective' eNOS pool localizes in endothelial cells, cardiac myocytes, or any additional cell type(s). Despite

the lack of cell-specific genetic deletion experiments to settle this issue (which remains for all cardiac NOS), data obtained from 'tissue-specific' transgenic animals at least provided useful hints. Consistent with abundant localization of eNOS in vascular endothelium, Jones *et al.* reported that MI-induced congestive HF was attenuated in mice with endothelium-restricted eNOS overexpression.⁴⁶ Several studies provided evidence that cardiomyocyte-restricted overexpression of eNOS under the control of the α -myosin heavy chain promoter (α MHC-eNOS_{tg}) could also protect against adverse remodelling after TAC⁴⁷ or MI,⁴⁸ although fibrosis was not improved.⁴⁸ Of note, a recent study using isolated single myocytes also demonstrated that eNOS is the main source of constitutively produced NO (i.e. in the absence of inflammatory stimuli) in response to angiotensin II, a typical mediator of remodelling.⁴⁹ This, together with the fact that eNOS co-localizes with specific downstream effectors confined to specific subcellular domains in cardiac myocytes (see following section 3.1), highlights eNOS-derived NO both as a critical regulator of remodelling and as an attractive therapeutic target to prevent and/or treat post-ischaemic remodelling and HF.

However, the efficiency of eNOS (as all NOS) signalling depends on the balance between its 'coupled' (in dimeric form, producing NO) vs. 'uncoupled' (monomeric, producing superoxide anion) status (see section 1, above), with the latter producing deleterious effects during conditions of heavy, long-term haemodynamic overload.⁵⁰ Interestingly, BH₄ supplementation restored the protective effect of NOS in the context of severe cardiac remodelling, and this mechanism was shown to act specifically on cardiac eNOS but not endothelium-derived eNOS from heart blood vessels.⁵⁰ The effect of BH₄ supplementation may not be restricted to eNOS, because iNOS-derived superoxide generation was reduced (and NO bioavailability increased) by BH₄ in a model of I/R injury in perfused hearts from diabetic rats.⁵¹ Thus, promoting the 'coupled' status of NOS activity in cardiac myocytes by BH₄ supplementation offers an interesting therapeutic approach (see section 4.2 below) and justifies further study of the mechanism for both eNOS activation and downstream regulation of pathological remodelling.

3. NOS-mediated pathways influencing remodelling

3.1 The NO–cGMP–PKG axis

Substantial evidence points to the soluble guanylyl cyclase (sGC)–cGMP–PKG pathway, a classic target of NO, as a regulator of stress-induced cardiac remodelling downstream of NOS. Although most of the *in vivo* data used models of pressure overload subsequent to thoracic aortic banding, similar mechanisms are probably also involved in post-ischaemic remodelling, during haemodynamic stress resulting from the loss of functional myocardium.⁵² Initial *in vitro* data showed that the NO donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine, the cGMP analogue 8-Bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP),⁵³ and adenoviral overexpression of PKG1⁵⁴ exert anti-hypertrophic effects on catecholamine-stimulated cardiomyocytes. *S*-nitroso-*N*-acetyl-D,L-penicillamine and 8-Br-cGMP also prevented the proliferation of cardiac fibroblasts.⁵³ These effects could be at least partly mediated by inhibition of the classic Ca²⁺-sensitive pro-hypertrophic calcineurin/nuclear factor of activated T cells (NFAT) signalling pathway (recently reviewed in reference⁸). Consonant with these *in vitro* data, the preferential PDE5 inhibitor, sildenafil,

not only prevented but also reversed TAC-induced adverse remodeling and the activation of pro-hypertrophic pathways in mice. However, as cGMP is also produced by the particulate GC (GC-A) upon binding of natriuretic peptides, NOS activity may not be the only source of cGMP mediating protection. Cardiac-restricted deletion of GC-A showed that natriuretic peptide mediates an anti-hypertrophic action in the heart, independent from its role in regulating blood pressure. Thus, sGC-dependent and GC-A-dependent pools of cGMP may converge on some common pathways regulating hypertrophy, including Calcineurin (Cn)-NFAT. Nevertheless, the sildenafil-sensitive pool of cGMP that regulates cardiac inotropy seems to be orchestrated by eNOS and not by natriuretic peptides.⁵⁵ Taken together, these data suggest a mechanism whereby eNOS-derived NO would activate sGC and generate a subcellularly confined pool of cGMP, leading to activation of a subset of PKG1 and upstream attenuation of signalling pathways involved in hypertrophy.

There may be several relevant targets of PKG1 in the context of stress-induced remodelling.⁸ Protein kinase G may alter the functional properties of several effectors of E–C coupling, with subsequent modifications of Ca^{2+} handling and contractility that, in turn, could modulate remodelling. One potential phosphorylation target is phospholamban on its Ser-16 residue, resulting in increased sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) activity and diastolic Ca^{2+} uptake. Accordingly, increased SERCA activity was shown to reduce hypertrophy, fibrosis, and mortality in mice with increased cardiac β_1 -adrenergic activity, probably by restoring the normal diastolic Ca^{2+} level.⁵⁶ Such an effect, however, would have to be balanced with PKG-mediated reduction in phosphorylation of the L-type Ca^{2+} channel or direct troponin I phosphorylation that would depress the inotropic state; whether this confers further protection during sustained β -adrenergic stimulation or worsens the development of HF still needs to be examined in the long term.

The importance of Ca^{2+} handling was recently highlighted by the identification of effectors implicated in stress-induced hypertrophy, either as activators [L-type Ca^{2+} channels, transient receptor potential cation channel, subfamily C, member 6 (TRPC6), transient receptor potential cation channel, subfamily C, member 3 (TRPC3), T-type Ca^{2+} channel $\alpha 1\text{H}$ (Cav3.2) subunit] or as inhibitors [plasma membrane Ca^{2+} ATPase isoform 4b, T-type Ca^{2+} channel $\alpha 1\text{G}$ subunit (Cav3.1)]. Notably, these data converge on the notion that hypertrophic pathways are regulated by small, compartmentalized Ca^{2+} fluxes,⁵⁷ functionally independent from the larger, cyclic Ca^{2+} waves governing contractility. In this context, the co-localization of eNOS and (a subset of) sGC and PKG in the vicinity of some of these Ca^{2+} handling effectors probably has functional relevance. Indeed, *in vitro* data showed that the phosphorylation of TRPC6 by PKG (stimulated with 8-Br-cGMP or sildenafil, which specifically affects the eNOS-dependent cGMP pool)⁵⁵ can prevent TRPC6-mediated NFAT activation.⁵⁸

Moreover, a direct association was shown between eNOS and the $\alpha 1\text{G}$ T-type Ca^{2+} channel that conferred protection from adverse remodelling *in vivo*.⁵⁹ The channel subunit co-immunoprecipitated with eNOS, and inhibition or deletion of eNOS abrogated the protective effect conferred by inducible, cardiac-specific overexpression of the $\alpha 1\text{G}$ protein. Protein kinase G activity was also increased in heart extracts from these transgenic mice after pressure overload. The fact that both eNOS (also a Ca^{2+} -sensitive enzyme) and the $\alpha 1\text{G}$ T-type Ca^{2+} channel are known caveolae residents further points to NOS (and possibly, PKG)-dependent regulation of Ca^{2+} handling

effectors and hypertrophic remodelling in specific cellular microdomains.

Other (Ca^{2+} -unrelated) PKG targets could also regulate hypertrophy. Among these, the regulator of G-protein signalling-2, which is known to attenuate the transduction of G_q signalling, was shown to attenuate pressure overload-induced hypertrophy and to mediate the anti-hypertrophic effects of sildenafil *in vivo*. In addition to regulating hypertrophy of cardiomyocytes, the cGMP–PKG axis may influence other important aspects of remodelling, such as angiogenesis and fibrosis, thereby extending the potential benefits associated with activation/restoration of NOS activity.

Recently, the pivotal role of PKG1 in attenuating pressure overload (or neurohormone)-induced hypertrophy was questioned, based on the phenotype of mice with genetic ablation of PKG1.⁶⁰ As the systemic deletion is lethal, a first model used 'rescue' PKG1 α expression under a smooth muscle cell promoter on a PKG1^{−/−} background, leaving other cells, including cardiomyocytes, depleted of the enzyme. These mice did not exhibit enhanced hypertrophy in response to catecholamines or TAC; however, aside from potential problems with the model itself, the stress imposed on the mice in this study may have been too mild to fully activate PKG and/or its known targets mediating the hypertrophic response, e.g. NFAT and Ca^{2+} –calmodulin-dependent kinase.⁶⁰ Indeed, in other mouse models with either expression of a mutant form of PKG1 α (unable to bind effectors)⁶¹ or with cardiomyocyte-restricted deletion of PKG1 α ,⁸ TAC did produce a worse remodelling, with deterioration of cardiac function. Likewise, although sildenafil may target PDE1 in addition to PDE5 in some species (e.g. humans), its cGMP-potentiating and protective effect was shown to depend on PKG1 α expression, at least in mice.⁶¹ Finally, the possibility that the cGMP-stimulated PDE2 mediates the effects of sildenafil upon catecholamine (i.e. G_s -coupled isoprenaline) stimulation seems unlikely, given that PDE2 inhibition had no influence on the sildenafil-induced negative inotropic effect upon isoprenaline stimulation.⁶² Again, this points to specific signalling modulation by spatially confined pools of cGMP.

3.2 cGMP-‘independent’ protective effects of NO

In addition to the cGMP axis, NO modulates many effector proteins through nitrosylation, i.e. the post-translational covalent modification of a protein cysteine thiol by a NO group to generate S-nitrosothiols (for a review see reference⁶³). S-nitrosylation was shown to regulate numerous functions in cardiovascular tissues, including vascular tone, angiogenesis, inflammation, apoptosis, E–C coupling, and G-protein-coupled receptor signalling. In the context of myocardial injury or failure, the production of reactive oxygen species, e.g. superoxide anions from mitochondria, NADPH and xanthine oxidase activities may alter NO biological signalling by directly reacting with NO (to produce peroxynitrite, ONOO[−]), competing with NO for irreversible, covalent binding with cysteine thiols, and by directly limiting NO synthesis by NOS oxidation and uncoupling.⁶ Maladaptive remodelling may therefore be the consequence of the decreased bioavailability/bioactivity of NO produced by NOS in specific compartments where it normally regulates cardiac and coronary functions via protein S-nitrosylation. Such nitroso-redox imbalance has been observed in hypertensive rats with HF, where global nitrosylation of myocardial proteins is reduced and can be restored by

treatment with oxypurinol, a xanthine oxidase inhibitor.⁶⁴ In this context, the protection conferred by nNOS against post-MI remodelling (see previous section 2.1) could be due to its inhibition of XOR,⁶⁵ as reproduced by cardiac overexpression of nNOS in I/R injury.²¹

Importantly, S-nitrosylation is a reversible and dynamic process. S-nitrosogluthathione reductase (GSNOR) is a key enzyme for de-nitrosylation.⁶⁶ An important insight on the extent of cardioprotection conferred by S-nitrosylation was provided by a study of GSNOR knockout mice submitted to MI. These mice with enhanced S-nitrosylated proteins show reduced myocardial infarct size, preserved ventricular systolic and diastolic function, and blunted eccentric remodelling 12 weeks after MI. Constitutive S-nitrosylation of hypoxia-inducible factor was proposed as a central (albeit not exclusive) mechanism for this protection.⁶⁷ Using a new resin-assisted capture technique, Kohr *et al.*⁶⁸ recently studied the dynamics of nitrosylated and oxydized thiol residues in proteins from heart tissue following I/R preceded or not by ischaemic preconditioning. They showed that the amount of nitrosylated proteins was increased by preconditioning, and that the degree of oxidation of this particular protein pool was lower than in non-nitrosylated proteins. This led them to propose that initial, transient nitrosylation could act as a 'shield', preventing protein thiols from subsequent oxidation during the reperfusion phase.

Several protein targets have been identified for nitrosylation by endogenous NOS that could account for their protective effects. A recent study⁶⁹ showed that the elevated systolic and diastolic $[Ca^{2+}]_i$ and L-type Ca^{2+} channel currents in $NOS1^{-/-}$ cardiomyocytes were accompanied by decreased nitrosylation of important effectors of Ca^{2+} handling proteins, such as L-type Ca^{2+} channel $\alpha 1C$ subunit, ryanodine receptor 2 and SERCA2a, consistent with previous evidence for their regulation by nitrosylation (reviewed in reference⁶³). For example, nitrosylation of the L-type Ca^{2+} channels diminishes Ca^{2+} influx^{70–72} and is enhanced after I/R via eNOS and nNOS activities.^{71,72} This could be facilitated by co-localization of the channel with both enzymes into caveolae. Likewise, nitrosylation of the cardiac ryanodine receptor 2 was shown to increase its open probability.² Limited, regulated nitrosylation of ryanodine receptor 2 by eNOS may underlie the slow increase in calcium and contractile force (i.e. the Anrep effect) in isolated mouse and rat cardiomyocytes in response to stretch.⁴¹ Conversely, decreased nitrosylation observed in $NOS1^{-/-}$ (but not $NOS3^{-/-}$) animals is accompanied by Ca^{2+} leak and arrhythmogenic Ca^{2+} waves, possibly attributable to unopposed XOR activity and nitroso-redox imbalance.⁷³ Other protein targets for S-nitrosylation include signal transduction elements of G-protein-coupled receptors (e.g. β -adrenergic, reviewed in reference⁶³). S-nitrosylation of phospholamban was described in fish hearts,⁷⁴ but with no equivalent in mammals so far. Finally, S-nitrosylation also regulates energy metabolism (particularly through mitochondrial targets),⁶⁸ although the implications for post-ischaemic remodelling need further study. Spatial confinement of eNOS or nNOS in the normal heart or their translocation in pathophysiological situations probably guarantees both efficient and specific S-nitrosylation of these effectors; co-localization of the NOS with GSNOR would probably add another layer of regulation, but has been little studied so far.

Combination of oxidative stress and NO production can also lead to modifications of lipids through formation of biologically active nitro-fatty acids.⁷⁵ The relevance of nitro-fatty acids in cardiac pathophysiology was suggested by a recent *in vivo* study showing that mice having undergone I/R have increased levels of nitro-oleic acid and

nitro-linoleic acid compared with control animals.⁷⁶ Interestingly, administration of nitro-oleic acid reduced the severity of the reperfusion-induced injury and inflammatory reaction, and slightly improved fractional shortening. Such data add further complexity to the potential biological effects of NO and oxidant radicals but also suggest interesting new therapeutic directions that deserve further investigation.

4. Translational perspectives of NOS modulation in post-ischaemic remodelling

The above sections highlight the growing weight of evidence in favour of the causality of cardiac NOS in driving adaptive (with fully functional NOS) vs. maladaptive remodelling (with dysfunctional NOS). The following paragraphs examine how this knowledge can be translated into therapeutic approaches by targeting specific cardiac NOS and/or downstream pathways.

4.1 Phosphodiesterase 5A inhibitors and soluble guanylyl cyclase activators

As discussed above (section 3.1), convincing evidence supports the benefit of strategies reinforcing the NO–cGMP–PKG1 axis to limit adverse post-ischaemic cardiac remodelling. Indeed, the abundance of the cGMP-hydrolysing PDE5 protein was increased in hearts from patients with advanced systolic HF due to ischaemic or dilated cardiomyopathy in comparison with heart tissue from control patients,⁷⁷ and transgenic mice with cardiac-restricted PDE5 overexpression were predisposed to adverse left ventricular remodelling with reduced contractile reserve after myocardial infarction. This provides a rationale for the therapeutic use of PDE5 inhibitors or sGC activators to preserve the cGMP pool and the associated protective effects. The relevance of PDE5 as the main (or exclusive) cGMP-hydrolysing enzyme in the heart is, however, debatable, because PDE1 may also be involved and is inhibited by sildenafil at high doses.⁷⁸ Data comparing cGMP-hydrolysing activities confirmed the importance of PDE5 in normal and failing mouse hearts but less so in failing human myocardium. This stresses the need for a thorough characterization of cGMP-hydrolysing activities in human disease, in order to target the most relevant PDE isoform with specific inhibitors. Nevertheless, sildenafil is currently being tested in a National Institutes of Health sponsored clinical trial of diastolic HF (Evaluating the effectiveness of sildenafil at improving health outcomes and exercise ability in people with diastolic heart failure – The RELAX Study).

The direct sGC activator BAY 58-2667 showed protective properties by reducing infarct size in perfused rodent hearts submitted to I/R,⁷⁹ and this protection was blunted by an inhibitor of PKG (KT 5823; 1 μ M) or the blocker of the mitochondrial ATP-sensitive potassium channel (5-hydroxydecanoate; 100 μ M), a possible cardioprotective target of PKG.⁸⁰ BAY-58-2667 also demonstrated benefits clinically in patients with acute decompensated HF through reduction in cardiac loading, improved cardiopulmonary haemodynamics, and cardiac output.⁸¹ A parent compound, riociguat (BAY 63-2521), markedly attenuated systemic hypertension, systolic dysfunction, and fibrotic myocardial remodelling, as well as improving survival in a rodent model of pressure and volume overload.⁸²

4.2 BH₄ supplementation and folic acid

As mentioned above (section 2.3), during harsh haemodynamic stress, the myocardial NOS can be uncoupled, and the resulting increased oxidant stress participates in maladaptive remodelling. Depletion in reduced BH₄ is one factor of NOS uncoupling, justifying the potential benefit of BH₄ supplementation, if properly dosed.⁵⁰ Further investigation is needed to establish the clinical proof of concept for the therapeutic efficacy of such a strategy in ischaemic and failing cardiac diseases.

Folic acid (or vitamin B9) has shown protective effects on cardiovascular tissues through homocysteine regulation and increases in eNOS activity. Folic acid can replenish reduced BH₄ levels⁸³ and therefore help to maintain eNOS in its coupled state. Encouraging clinical results support the proposition that folic acid supplementation can improve endothelial function in patients with various cardiovascular diseases. For example, 6 weeks of treatment with high-dose folic acid improved endothelial function in post-acute MI patients.⁸⁴ This is in line with experimental studies showing myocardial protection and preserved function in rats submitted to MI or I/R when folic acid was administrated.⁸⁵

4.3 The β_3 -adrenergic pathway

In the human and mouse ventricle, β_3 -adrenergic receptors (ARs) exert an inotropic influence that is mediated by eNOS activation and functionally antipathetic to that of β_1 - and β_2 -adrenergic stimulation.^{86,87} This suggests the importance of β_3 -ARs for eNOS-mediated blunting of the cardiac effects of neurohormones. Moreover, a similar inhibition of the β_1 - and β_2 -adrenergic inotropic effect by sildenafil in mouse cardiomyocytes was recently shown to be mediated by the β_3 -AR.⁶² Therefore, several pieces of evidence converge to the involvement of β_3 -ARs to stimulate, via eNOS, the production of a cGMP pool that is sensitive to sildenafil in the context of cardiac stress, e.g. with neurohormonal stimulation. Additional facts would reinforce the interest in such possibility (for reviews, see references^{88,89}), as follows: (i) in contrast to β_1 - and β_2 -adrenergic receptors, the expression of β_3 -ARs was shown to increase in patients with ischaemic and dilated cardiomyopathies;⁹⁰ (ii) the β_3 -AR lacks consensus phosphorylation sites involved in receptor desensitization; (iii) although acute stimulation of β_3 -ARs attenuates contractile shortening of ventricular muscle *in vitro*,⁸⁶ chronic overexpression of β_3 -ARs *in vivo* does not result in contractile dysfunction or decreased contractile reserve.^{91,92} Interestingly, mice with systemic deletion of the β_3 -AR develop age-dependent cardiac hypertrophy. Moreover, β_3 -AR knockout mice showed worse remodelling than wild-type mice following TAC, including enhanced fibrosis, cardiomyocyte hypertrophy, and LV dilatation.⁹³ Reciprocally, data from our group⁹¹ show that transgenic mice with cardiac-restricted β_3 -AR overexpression are protected from cardiac hypertrophy induced by chronic infusion of neurohormones, and that this protection is reversed by NOS inhibition. Further data recently showed that the protection against I/R injury and increased phosphorylation of Ser-1177 of cardiac eNOS conferred by voluntary exercise were lost in β_3 -AR^{-/-} mice.⁹⁴ Acute administration of the preferential β_3 -AR agonist, BRL37344, was recently reported to decrease contractility slightly (assessed by pressure–volume loop analysis) in non-failing hearts of normal sheep but, strikingly, it improved LV function in post-ischaemic failing hearts.⁹⁵ Mechanistically, this was attributed to β_3 -AR stimulation of Na⁺–K⁺-ATPase activity by reducing

glutathionylation of the β Na⁺–K⁺ pump subunit, through a NOS-dependent mechanism. Whether this would also prevent adverse myocardial remodelling upon chronic β_3 -AR stimulation still remains to be studied.

There is only indirect evidence, so far, that such protective effects may be relevant to human HF. One such piece of evidence is the result from the SENIORS trial,⁹⁶ which established the benefit of nebivolol in an elderly population of patients with HF. Nebivolol is endowed with dual β_1 -AR blockade and β_3 -AR agonistic properties, as demonstrated *ex vivo* in human microcoronary arteries⁹⁷ and human non-failing myocardium.⁹⁸ Although superiority compared with a 'pure' β_1 -AR blocker was not demonstrated, at least it suggests that the additional β_3 -AR stimulation may not be harmful. A direct comparison of nebivolol with the β_1 -AR antagonist, metoprolol, in post-MI remodelling was recently performed in mice and demonstrated a superior efficacy of nebivolol to preserve LV ejection fraction and survival 4 weeks after MI.⁹⁹ Notably, these additional benefits were lost in NOS3^{-/-} mice, again pointing to a mechanistic role of eNOS-derived NO.

5. Conclusion

After the original descriptions of the functional role of the cardiac nitric oxide synthases,^{100–102} the last decade has seen a tremendous amount of information contributed from experimental research confirming their expression, regulation, and signalling to specific pathways regulating myocardial remodelling; this involves both cGMP-dependent and independent effectors, whose role is increasingly being deciphered from the phenotype of genetically modified mouse models. These data, together with analysis at the cardiomyocyte level, emphasize the specialized, non-redundant role of each cardiac NOS, subserved by their spatial confinement and co-localization with specific targets that ensure signalling specificity. Accordingly, pools of cGMP have been identified, with non-overlapping function in their regulation of E–C coupling or hypertrophy. Such compartmentation is particularly relevant for the regulation, by the cardiac NOS, of the nitroso-redox balance that is key for cellular homeostasis in the face of ischaemic, inflammatory, or other pro-oxidant stresses. Future research will probably expand on the molecular characterization of signalosomes around the NOS, including upstream G-protein-coupled receptors or other receptors and associated regulators/effectors; among these, it will be interesting to dissect the molecular determinants of the subcellular localization of effectors, such as PKG1 in cardiac myocytes, or of GSNOR that may preferentially regulate S-nitrosylation of NOS targets. Likewise, the biochemical machinery known to regulate NOS 'coupled' catalytic activity in vascular tissue has been very little studied in the context of cardiac myocytes so far, but should receive more attention, given the emerging role of the nitric oxide synthases as gatekeepers of reactive oxygen species-mediated myocardial dysfunction and remodelling. Hopefully, this should expand the current list of therapeutic options to prevent/reverse post-ischaemic cardiomyopathies through more efficient harnessing of cardiac NOS signalling.

Conflict of interest: none declared.

Funding

Work supported by PAI P6-30 of 'Politique Scientifique Fédérale', Action de Recherche Concertée of 'Fédération Wallonie-Bruxelles' and the

Fonds National de la Recherche Scientifique (FNRS); V.M. is Clinicien-Chercheur of the FNRS.

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