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SYMPOSIUM REVIEW

Molecular mechanisms of neuronal nitric oxide synthase in cardiac function and pathophysiology

Yin Hua Zhang[1,2,3], Chun Zi Jin[3], Ji Hyun Jang[1] and Yue Wang[1]

[1] *Department of Physiology, College of Medicine, Seoul National University, Seoul, South Korea*
[2] *Ischaemic/Hypoxic Disease Institute, College of Medicine, Seoul National University, Seoul, South Korea*
[3] *Clinical Research Center, Yanbian University Hospital, Yanji, Jilin Province, China*

Abstract Neuronal nitric oxide synthase (nNOS or NOS1) is the major endogenous source of myocardial nitric oxide (NO), which facilitates cardiac relaxation and modulates contraction. In the healthy heart it regulates intracellular Ca^{2+}, signalling pathways and oxidative homeostasis and is upregulated from early phases upon pathogenic insult. nNOS plays pivotal roles in protecting the myocardium from increased oxidative stress, systolic/diastolic dysfunction, *adverse* structural remodelling and arrhythmias in the failing heart. Here, we show that the downstream target proteins of nNOS and underlying post-transcriptional modifications are shifted during disease progression from Ca^{2+}-handling proteins [e.g. PKA-dependent phospholamban phosphorylation (PLN-Ser^{16})] in the healthy heart to cGMP/PKG-dependent PLN-Ser^{16} with acute angiotensin II (Ang II) treatment. In early hypertension, nNOS-derived NO is involved in increases of cGMP/PKG-dependent troponin I (TnI-$Ser^{23/24}$) and cardiac myosin binding protein C (cMBP-C-Ser^{273}). However, nNOS-derived NO is shown to increase *S*-nitrosylation of various Ca^{2+}-handling proteins in failing myocardium. The spatial compartmentation of nNOS and its translocation for diverse binding partners in the diseased heart or various nNOS splicing variants and regulation in response to pathological stress may be responsible for varied underlying mechanisms and functions. In this review, we endeavour to outline recent advances in knowledge of the molecular mechanisms mediating the functions of nNOS in the myocardium in both normal and diseased hearts. Insights into nNOS gene regulation in various tissues are discussed. Overall, nNOS is an important cardiac protector in the diseased heart. The dynamic localization and various mediating mechanisms of nNOS ensure that it is able to regulate functions effectively in the heart under stress.

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Corresponding author Y. H. Zhang: Department of Physiology, Seoul National University, College of Medicine, 103 Dae Hak Ro, Chong No Gu, 110-799 Seoul, South Korea. Email: yinzhang87@gmail.com

Abbreviations eNOS, endothelial nitric oxide synthase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; iNOS, inducible nitric oxide synthase; LTCC, L-type Ca^{2+} channel; L-VNIO, N5-(1-imino-3-butenyl)-L-ornithine; 7-NI, 7-nitroindazole; NMDAR, *N*-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NOSIP, nitric oxide synthase interacting protein; PFK, phosphofructokinase; PIN, protein inhibitor of nNOS; PLN, phospholamban; RNS, reactive nitrogen species; ROS, reactive oxygen species; RYR, ryanodine receptors;

Dr. Yin Hua Zhang is Associate Professor at Seoul National University, College of Medicine, where she was trained as a Cardiac Electrophysiologist with Professor Yung E. Earm. She received further training in Bristol University with Professor Jules Hancox and in Oxford University with Professor Barbara Casadei. Her current research projects involve molecular mechanisms mediating cardiac protection from failing during disease progression (hypertension & metabolic syndrome). In addition, her team aims to reveal novel targets of nitric oxide and reactive oxygen species in regulating myocyte Ca^{2+} homeostasis, myofilament proteins, metabolism and contraction in the myocardium of cardiac arrhythmias and heart failure.

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Introduction

Nitric oxide (NO) has been identified as an important signalling molecule involved in a broad range of biological functions since the late 1970s (Arnold *et al.* 1977; Gruetter *et al.* 1979; Furchgott & Zawadzki, 1980), a discovery that led Robert F. Furchgott, Louis J. Ignarro and Ferid Murad to win the Nobel Prize in Physiology or Medicine in 1998. It is now clear that NO-producing enzymes, NO synthases (NOSs), are specified by three genes: neuronal NOS (nNOS or *NOS1*); inducible NOS (iNOS or *NOS2*), and endothelial NOS (eNOS or *NOS3*). Until recently, eNOS has been considered the only isoform of NOS in the myocardium and its regulation of a variety of functions in the heart is well documented (Shah & MacCarthy, 2000; Massion *et al.* 2003). In 1999, Xu *et al.* (1999) demonstrated that nNOS is constitutively expressed in the sarcoplasmic reticulum (SR) of cardiac myocytes and regulates sarcoplasmic Ca^{2+} ATPase (SERCA) reuptake of intracellular Ca^{2+}. nNOS is also the only NOS isoform that is expressed in intrinsic cardiac neurons from autonomic nerves and ganglions (Mohan *et al.* 2000; Choate *et al.* 2001; Danson *et al.* 2005) and controls parasympathetic and sympathetic regulation of cardiac rhythm and contractility. Furthermore, nNOS is expressed in human coronary artery smooth muscle cells (Han *et al.* 2007) and maintains the basal blood flow (Seddon *et al.* 2009). Taken together, these findings confirm that nNOS is an important NOS in the heart that is expressed in all the vital fractions and plays important roles in regulating the rhythm, contractility and microcirculation of the heart (Fig. 1*A*).

Neuronal NOS shares similarities with eNOS in structure and the logistics of NO production. Active nNOS is a homodimer in which each monomer is composed of an N-terminal oxygenase domain and a C-terminal reductase domain (Fig. 1*B*). NO production takes place in the oxygenase domain by catalysing L-arginine to L-citruline [endorsed by O_2 as the other substrate in the presence of cofactor tetrahydrobiopterine (BH4) and electron transfer from nicotinamide adenine dinucleotide phosphate (NADPH) *via* the flavin adenine dinucleotide (FAD)–flavin mononucleotide (FMN) axis to the heme iron in the oxygenase domain of the other monomer]. Ca^{2+}-calmodulin is essential in linking FMN and heme iron to ensure efficient electron transfer by facilitating FMN donation of electrons and the alignment of the FMN and heme domains (Masters *et al.* 1996; Feng *et al.* 2014) (Fig. 1*B*). In cardiac myocytes, nNOS differs from eNOS in localization and translocation upon stimulation, post-transcriptional modification and the mechanisms mediating physiological and pathological functions in the cardiovascular system (Alderton *et al.* 2001; Massion *et al.* 2003; Belge *et al.* 2005; Zhang & Casadei, 2012). For example, eNOS is primarily located in caveolae (Feron *et al.* 1996) and mediates mechanical stress stimulation of intracellular Ca^{2+} release from ryanodine receptors (RyR) (Petroff *et al.* 2001). nNOS is located in the SR (Xu *et al.* 1999; Bendall *et al.* 2004) and in plasma membrane (Ueda *et al.* 2008) and regulates Na^+ and Ca^{2+} homeostasis in cardiac myocytes. nNOS-derived NO modulates myocyte contraction and facilitates relaxation by targeting key elements of excitation–contraction coupling [e.g. phospholamban (PLN) or L-type Ca^{2+} channels (LTCC)]. This is understandable because NO has a short half-life (~10 s) and limited diffusion area (due to abundant NO scavenging myoglobin and NO-interacting biochemicals and proteins); thus the compartmentation of nNOS or the translocation of nNOS to the vicinity of target proteins seem essential to its competent effects. In essence, the spatial confinement of nNOS and its dynamic translocation in response to stimuli ensure that it is able to regulate diverse intracellular signalling pathways and myocardial functions in normal and diseased hearts.

The function and mechanisms of nNOS regulation in the heart

nNOS in the healthy heart. Using gene knockout of nNOS (Barouch *et al.* 2002; Sears *et al.* 2003; Dawson *et al.* 2005; Saraiva *et al.* 2005) and cardiomyocyte-specific nNOS overexpression models (Burkard *et al.* 2007; Loyer *et al.* 2008; Burkard *et al.* 2010) in conjunction with nNOS inhibitors [e.g. *S*-methyl-L-thiocitrulline (SMTC), N5-(1-imino-3-butenyl)-L-ornithine (L-VNIO) or 7-nitroindazole (7-NI)] (Xu *et al.* 1999; Sears *et al.* 2003; Bendall *et al.* 2004; Seddon *et al.* 2009), it has been established that NO produced from constitutive nNOS provides an intrinsic regulatory mechanism of myocardial contraction and relaxation in both the healthy and the diseased heart. In the healthy heart, nNOS-derived NO attenuates basal cardiac inotropy by modulating the activities of LTCC in the plasma membrane to reduce the amplitude of intracellular Ca^{2+} transients (Sears *et al.* 2003) through *S*-nitrosylation- or cGMP-dependent mechanisms. By contrast, the activity of the cardiac Na^+–Ca^{2+} exchanger is unaffected (Sears *et al.* 2003). In the SR, nNOS-derived NO facilitates myocyte relaxation by promoting SERCA reuptake of intracellular Ca^{2+} by increasing PKA-dependent (but cGMP/PKG-*independent*) PLN phosphorylation at serine16 (PLN-Ser^{16}) and Ca^{2+}-calmodulin-dependent kinase II (CaMKII)-dependent PLN phosphorylation at threonine17 (PLN-Thr^{17}), subsequent to the inhibition of cytosolic protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) activities by nNOS-derived NO (Zhang *et al.* 2008). nNOS-derived NO may activate SERCA either directly through *S*-nitrosylation (Burger *et al.* 2009) or indirectly through peroxynitrite-dependent *S*-glutathionylation (Adachi *et al.* 2004). The role of nNOS-derived NO in RyR activity is controversial because

nNOS gene deletion has been associated with both increased RyR leak (Gonzalez *et al.* 2007) and decreased open probability of RyR (Wang *et al.* 2010) (Fig. 2). In addition, nNOS forms a macrocomplex with the plasma membrane Ca^{2+} pump (PMCA4b) and cardiac Na^{+} channel (SCN5A) linked by α-syntrophin (SNTA1) and is tonically inhibited by PMCA4b. Dissociation of nNOS with PMCA4b in an *SNTA1* mutation liberates nNOS from inactivation and subsequently increases *S*-nitrosylation of SCN5A and late Na^{+} current. Under these conditions, nNOS is associated with SCN5A-dependent long QT syndrome (Ueda *et al.* 2008) (Fig. 2).

Accumulating evidence shows that nNOS-derived NO regulates cardiac function by targeting proteins other than Ca^{2+}-handling elements. For example, results reported by our group and others show that nNOS tonically controls the activities of constitutive cardiac oxidases such as xanthine oxidoreductase (Kinugawa *et al.* 2005; Idigo *et al.* 2012) and NADPH oxidase (Zhang *et al.* 2009; Jin *et al.* 2012) and moderates the levels of intracellular superoxide and reactive oxygen species (ROS). As ROS (e.g. hydrogen peroxide) and reactive nitrogen species (RNS) (e.g. peroxynitrite) target protein kinases/phosphatases (Brennan *et al.* 2006; Burgoyne *et al.* 2007; Kohr *et al.* 2009), nNOS regulation of ion channel activity and intracellular Ca^{2+}-handling proteins may be mediated by an array of post-transcriptional modifications such as NO-dependent *S*-nitrosylation, ROS-dependent oxidation and kinase/phosphatase-dependent phosphorylation. In essence, the genres of the target proteins of nNOS-derived NO determine the downstream post-transcriptional modifications. Furthermore, nNOS may affect myocardial function by regulating mitochondrial proteins. Indeed, nNOS-derived NO was reported to inhibit the mitochondria respiration chain, including complexes I, III and IV (Torres *et al.* 1995; Welter *et al.* 1996; Chouchani *et al.* 2013) and reduces mitochondrial

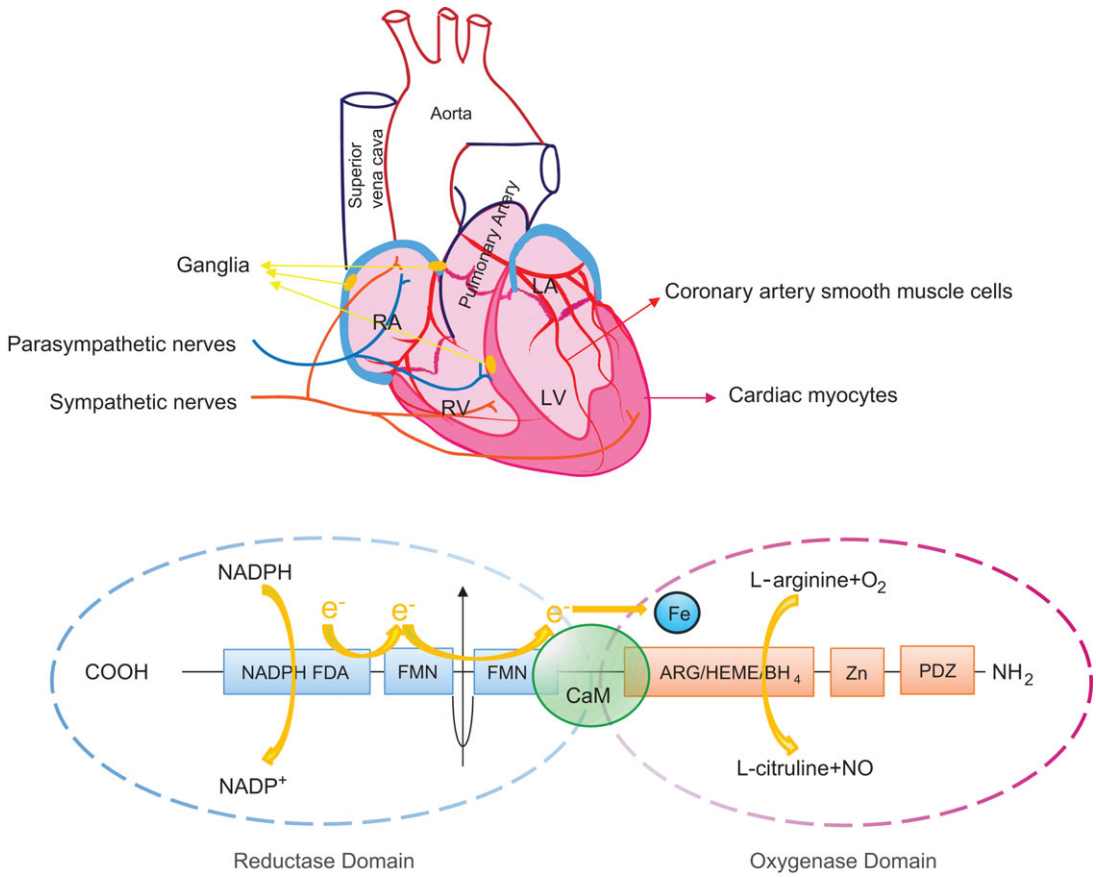

Figure 1. Distribution and structure of neuronal nitric oxide synthase (nNOS) protein in the heart
A, expression of nNOS protein in cardiac myocytes, coronary artery smooth muscle cells, cardiac ganglia, and sympathetic and parasympathetic nerves. *B*, structure of nNOS. Each monomer of nNOS contains an oxygenase domain (–COOH terminal) and a reductase domain (–NH2 terminal). Electrons from NADPH transfer from the reductase domain [*via* flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)] to the oxygenase domain (heme iron), enable nNOS to catalyse the oxidation of L-arginine to L-citrulline and release NO. These two domains are linked and the enzyme is activated by Ca^{2+}–CaM.

oxygen consumption and affects cardiac metabolism (Fig. 2). Whether such regulation is detrimental or beneficial in myocardial function in the healthy or diseased heart remains to be determined. Recently, nNOS was shown to be recruited by α-syntrophin to the nucleus membrane of murine skeletal muscle C2C12 cells and human HeLa cervix carcinoma cells and to be involved in mitochondrial biosynthesis by increasing the respiratory chain complexes through the promotion of cAMP response element-binding (CREB) protein *S*-nitrosylation-dependent activation of peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and its downstream oxidative phosphorylation genes *PGC-1α*, *NRF-1* and *mtTFA* (Aquilano *et al.* 2013). Whether similar mechanisms apply to the myocardium remains unknown. Overall, current evidence shows that nNOS-derived NO exerts cardiac functions by targeting essential proteins in various organelles in the myocardium.

nNOS in the diseased heart. Importantly, nNOS protein expression and activity are increased in the myocardium in the diseased heart, such as in ischaemia–reperfusion injury (Sun *et al.* 2006; Aragon *et al.* 2011), infarct (Takimoto *et al.* 2002; Bendall *et al.* 2004; Dawson *et al.* 2005; Burger *et al.* 2009), hypertrophy and heart failure (Damy *et al.* 2003, 2004; Niu *et al.* 2012), and exert manifold beneficial effects: nNOS-derived NO prevents diastolic dysfunction and increases β-adrenergic reserve, reduces left ventricular hypertrophy/dilatation/infarct size and protects the myocardium from arrhythmogenesis. In fact, recent findings by our group demonstrate that nNOS upregulation is an early event following pathogenic insult and during disease progression. We have shown that acute angiotensin II (Ang II, 1 μM) treatment to isolated left ventricular (LV) myocytes *in vitro* significantly increases mRNA/protein expressions and the activity of nNOS (Jin *et al.* 2012). In turn, nNOS-derived NO reduced NADPH oxidase production of superoxide and facilitated LV myocyte relaxation through cGMP/PKG-dependent (and PKA-*independent)* phosphorylation of PLN-Ser^{16} (Jin *et al.* 2012) (Fig. 3). Similarly, nNOS protein expression and activity were enhanced in LV myocytes from Ang II-induced *early* hypertensive rats (this is because Ang II infusion *in vivo* for 4 weeks did not

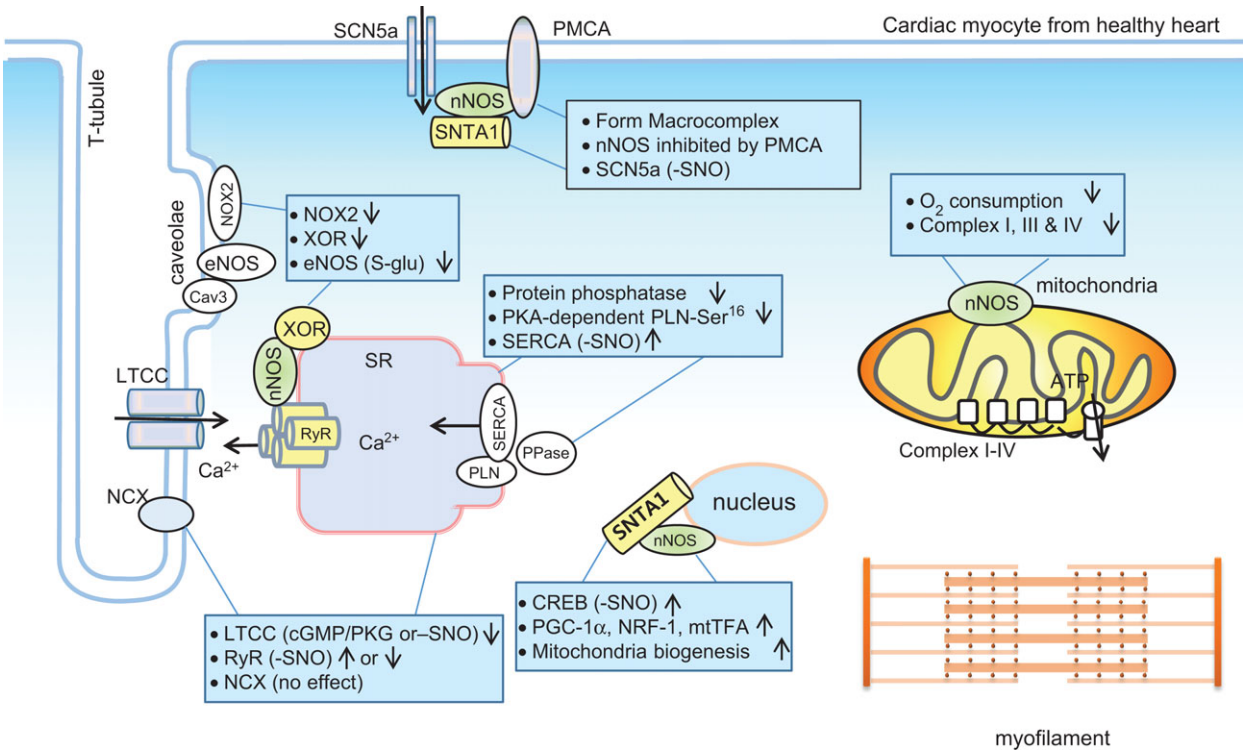

Figure 2. Neuronal nitric oxide synthase (nNOS) protein expression, compartmentation and function in healthy cardiac myocytes
The diagram illustrates the localization of nNOS and target proteins and regulation. nNOS is located in the sarcoplasmic reticulum (SR), mitochondria, plasma membrane and possibly nucleus. nNOS interacts with ryanodine receptor (RyR), xanthine oxidoreductase (XOR), α-syntrophin [forming a macrocomplex with Na^+ channel (SCN5a) and plasma membrane Ca^{2+}-ATPase (PMCA4b)]. nNOS modifies the activities of various proteins either through *S*-nitrosylation (-SNO) or through PKA-dependent, cGMP/PKG-dependent phosphorylations of downstream proteins.

develop hypertrophy and systolic/diastolic dysfunction detected using echocardiography). nNOS did not change contractility or LTCC activity, but did facilitate LV myocyte relaxation *via* myofilament Ca^{2+} desensitization mediated by cGMP/PKG-dependent phosphorylations of TnI-$Ser^{23/24}$ and cMyBP-C-Ser^{273} (Jin *et al.* 2013) (Fig. 3). Interestingly, PLN-Ser^{16} was increased and the kinetics of Ca^{2+} transient decay (Ca^{2+} reuptake *via* SERCA) were faster in LV myocytes from hypertensive rats; however, these changes were independent of nNOS or cGMP/PKG-dependent signalling (Jin *et al.* 2013). In line with these findings, nNOS protein expression was increased in the myocardium after 3 days and 6 days of Ang II infusion in rats (Tambascia *et al.* 2001; Moreno *et al.* 2002). These results demonstrate that nNOS upregulation is an early event during disease progression and nNOS plays protective roles in the heart under stress. In addition, the downstream targets of nNOS-derived NO are dynamic in order to facilitate effective function.

In stellate ganglia neurons, the protein expression and activity of nNOS are reduced and intracellular $[Ca^{2+}]_i$ are increased in pre-hypertensive (young spontaneously hypertensive) rats. Conversely, targeted nNOS transfer into sympathetic neurons using a novel noradrenergic cell-specific vector reduced $[Ca^{2+}]_i$ (Li *et al.* 2013). As nNOS is important in modulating sympathetic neural transmission (Paton *et al.* 2002), these results suggest that nNOS in the autonomic nervous system and contracting myocardium may coordinate to facilitate cardiac protective effects (e.g. the positive lusitropic effect) in early hypertension.

In human dilated cardiomyopathy and in the murine heart after myocardial infarction, nNOS upregulation is associated with reduced interaction with RyR and increased binding with caveolin-3 (Cav3) in plasma membrane of cardiac myocytes (Bendall *et al.* 2004; Sun *et al.* 2006). This location may potentiate its modulation of Ca^{2+} flux in plasma membrane or NADPH oxidase inhibition. Indeed, nNOS association with Cav3 is enhanced following ischaemia–reperfusion injury and nNOS attenuates β-adrenergic stimulation of I_{Ca} (by increasing *S*-nitrosylation of LTCC), and reduces the Ca^{2+} transient and SR Ca^{2+} contents, particularly in female mice (Sun *et al.* 2006). It has also been shown that nNOS protects the heart from ventricular arrhythmias after left coronary artery ligation by increasing *S*-nitrosylation and moderating the activities of key Ca^{2+}-handling proteins (e.g. LTCCα1c, SERCA2 and RyR2) and reducing Ca^{2+} influx, and diastolic and systolic Ca^{2+} transient amplitudes (Burger *et al.* 2009) (Fig. 4). Furthermore, nNOS exerts anti-arrhythmic effects by potentiating the peripheral vagal-induced modulation of heart rate (Choate *et al.* 2001; Heaton *et al.* 2005). In fact, stimulation of the vagal nerve increased nNOS production of NO in the

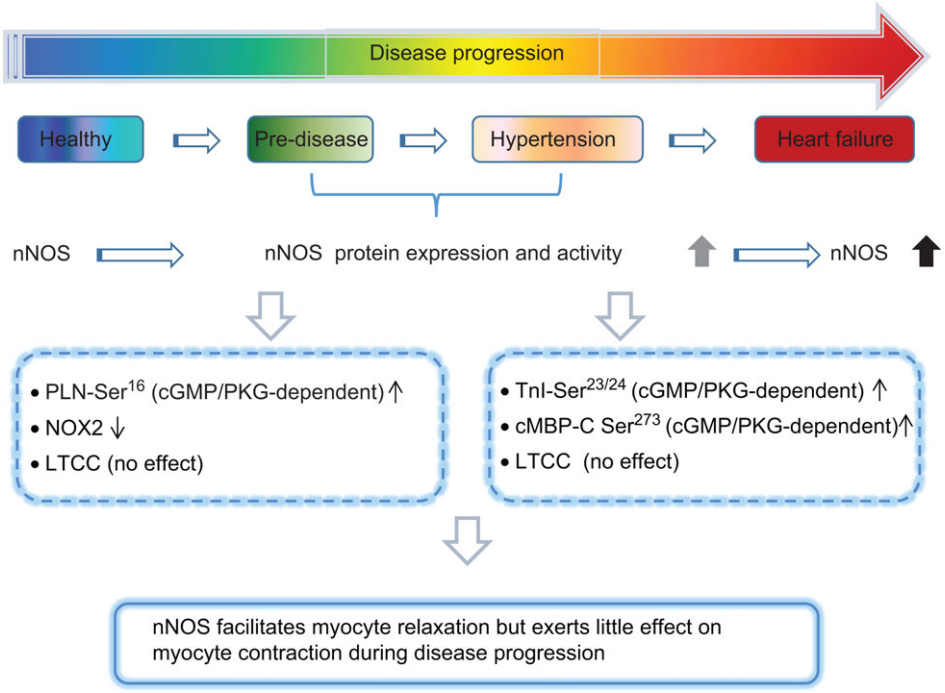

Figure 3. Cardiac neuronal nitric oxide synthase (nNOS) during disease progression
nNOS is functionally expressed in healthy myocardium. From the early stages of disease progression (pre-disease) to hypertension, cardiac nNOS is upregulated and facilitates myocyte relaxation. The mechanisms mediating nNOS shift from PKA-dependent PLN-Ser^{16} to PKG-dependent PLN-Ser^{16} at pre-disease and PKG-dependent cMBP-C Ser^{273} and cTnI $Ser^{23/24}$ at hypertension. Myocyte contraction is not affected by nNOS.

left ventricle and was anti-arrhythmic (Brack *et al.* 2009, 2011). Given that systemic sympathetic nervous activity is elevated in the diseased heart (Coote, 2005), vagal control of cardiac rhythm by nNOS appears to exhibit significant clinical relevance in the attenuation of fatal ventricular arrhythmias (Herring & Paterson, 2009).

The role of nNOS-dependent signalling in the mitochondria or myofilament in the diseased heart is not clear. In mice conditionally overexpressing nNOS, protein expression of nNOS is increased in mitochondria and myocardial availability of NO is increased. Consequently, nNOS reduced oxidative stress by attenuating mitochondria ROS and xanthine oxidoreductase (XOR) (Fig. 3) and attenuated infarct size following ischaemia–reperfusion (Burkard *et al.* 2010). Interestingly, depending on nNOS localization, downstream target proteins, mechanisms and functions, contrasting results occur in similar mouse models conditionally overexpressing nNOS (Burkard *et al.* 2007; Loyer *et al.* 2008). For example, overexpressed nNOS that is located predominantly in the SR increased LTCC activity and PLN-Ser^{16} and resulted in faster decay kinetics of $[Ca^{2+}]_i$ (Loyer *et al.* 2008). By contrast, overexpressed nNOS in the plasma membrane increased its interaction with LTCC, reduced LTCC activity and exerted little effect on PLN-Ser^{16} and, therefore, slowed the decay kinetics of $[Ca^{2+}]_i$ (Burkard *et al.* 2007). These results further strengthen the importance of spatial compartmentation of nNOS and its downstream target proteins in determining the phenotypes of diseased myocardium.

The nNOS gene, transcription and regulation

nNOS gene, multiple promoters/transcription and nNOS splice variants. The human nNOS or NOS1 gene is located on chromosome 12 (12q24.2) and the locus is dispersed over a region of 240 kb. The nucleotide sequence corresponding to the mRNA transcript is encoded by 29 exons and translates into a protein of 1434 amino acids with a predicted molecular weight of ≈160 kDa (Hall *et al.* 1994; Wang & Marsden, 1995). The nNOS gene has nine unique exon 1 variants for transcript initiation in different tissues and each transcript is expressed from a unique 5′-flanking region (Wang *et al.* 1999*b*); this makes the transcriptional/expressional regulation of *nNOS* extremely complex. In essence, diverse and structurally different nNOS mRNAs are initiated by a variety of transcriptional units *via* mechanisms including discrete promoters, alternative splicing, cassette exon deletions or insertions, and the usage of alternative polyadenylation signals (Wang *et al.* 1999*a*, *b*). In neurons and in muscle, *nNOS* promoters are clustered in genomic regions upstream from exon 2. Heterogeneous mRNAs from these promoters encoding the same nNOS proteins differ in both enzymatic characteristics and structural

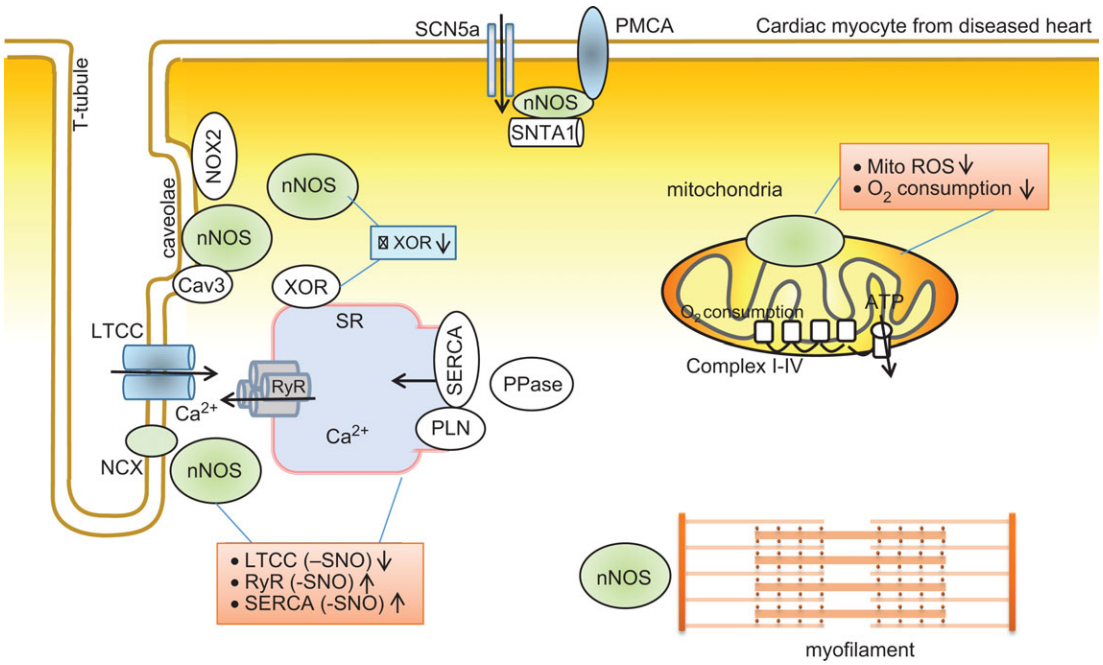

Figure 4. Neuronal nitric oxide synthase (nNOS) compartmentation, function and mechanism in failing cardiac myocyte
nNOS protein expression and activity are increased in failing myocardium. nNOS translocates to plasma membrane and interacts with caveolin-3 (Cav3) but dissociates from ryanodine receptors (RyR). nNOS-derived NO changes the activities of Ca^{2+} handling proteins *via* S-nitrosylation. Cardiac-specific overexpression of nNOS is associated with increased nNOS localization in mitochondria and moderates mitochondria activity.

features (Nakane *et al.* 1993; Xie *et al.* 1995; Young *et al.* 1995). The activation of promoters downstream of exon 2 results in the expression of transcripts that utilize alternative translation initiation sites and produce truncated forms of the protein (e.g. nNOSβ or nNOSγ); nNOSβ (but not nNOSγ) retains enzymatic activity but lacks a major PDZ protein–protein interaction domain, which is responsible for targeting nNOS to plasma or nucleus membranes (Eliasson *et al.* 1997). Different transcriptional initiation, processing and translational efficiency, stability and localization make nNOS transcripts susceptible to various stimuli and produce diverse isoforms. So far, five splice variants of nNOS are described (nNOSα, nNOSβ, nNOSγ, nNOSμ and nNOS2); however, little is known about the variants' specificity, functional relevance and mechanisms in cardiac myocytes. nNOSα and nNOSμ are known to contain PDZ domain and localize in plasma membrane linked with α-syntrophin. With a 34 amino acid insertion, nNOSμ differs from nNOSα in electron transfer rates, modulation of electron flow by CaM, and heme–nitrosyl complex formation (Panda *et al.* 2013). nNOSβ and nNOSγ do not contain PDZ domains and may be located in the cytosol.

nNOS gene regulation in tissues other than the myocardium. The genomic regulation (transcriptional and translational mechanisms) of cardiac nNOS is sparse, despite increased expression of nNOS mRNA and protein in LV or in atria following various pathophysiological insults (Takimoto *et al.* 2002; Damy *et al.* 2004; Danson *et al.* 2004; Ganzinelli *et al.* 2007). Understanding the transcriptional control of nNOS is important in defining the transcription/translation of specific nNOS splice variants in cardiovascular normal physiology and diseases. In neurons, skeletal muscle or vascular or gastrointestinal smooth muscle, nNOS transcription is regulated by transcriptional factors such as CREB (Sasaki *et al.* 2000), SP/ZNF families (Saur *et al.* 2002) or NF-kB (Li *et al.* 2007). CREB regulates the transcription of sirtuin in neuronal cells by binding to Sirt-1 chromatin; Sirt-1, in turn, is recruited by CREB to DNA and promotes CREB-dependent expression of nNOS (Fusco *et al.* 2012). Sequence analysis of 5′-flanking regions of the human nNOS gene revealed potential binding sites for AP-2, TEF-1/MCBF, CREB/ATF/cFOS, Ets, NF-1 and NF-kB-like sequences (Hall *et al.* 1994). All of the transcription factors are present in cardiac myocytes and therefore this information provides useful clues to the mechanism(s) involved in affecting nNOS gene regulation in cardiac myocytes.

Similarly, evidence of nNOS in tissues other than the heart may enhance understanding of nNOS splice variants and the functions in the myocardium. For example, it is well known that nNOSμ is upregulated after exercise in skeletal muscle in both animal models (Vassilakopoulos *et al.* 2003) and in humans (McConell *et al.* 2007). Expressions of nNOSμ mRNA and protein levels are correlated to angiogenesis after exercise (Huber-Abel *et al.* 2012). Recently, nNOSβ has been found to target Golgi and to play a pivotal role in maintaining the structure of murine skeletal muscle and post-exercise muscle strength, as well as in increasing resistance to muscle fatigue (Percival *et al.* 2010). These results suggest that the functional significance of nNOS splice variants refers to the regulation of diverse skeletal muscle functions. However, whether nNOS variants (nNOSα, nNOSβ and nNOSμ) play distinctive roles in cardiac myocytes is not yet clear. In the rat kidney cortex during late pregnancy, nNOSβ protein expression was increased, whereas nNOSα was reduced; consequently, antioxidant capacity was increased so that the renal cortex was protected from oxidative stress (Cunningham *et al.* 2013). Additionally, a high salt diet (from 0.4% to 4%) increased expression of nNOSβ protein to a greater extent than that of nNOSα in the inner medullary collecting duct of rat kidney (Hyndman *et al.* 2013). Similar changes in nNOSβ occur after right kidney removal and left kidney two-thirds ablation/infarction, and in kidney transplant models (Tain *et al.* 2008, 2011). By the same token, relaxin was found to increase nNOSβ expression in mouse proximal colon neurons, but to decrease nNOSα expression in smooth muscle cells, resulting in reduced muscle tone and increasing the amplitude of spontaneous muscle contraction (Baccari *et al.* 2012). The rise in nNOSβ was selectively suppressed by the β-adrenergic antagonist, nebivolol, and the angiotensin II type 1 receptor antagonist, olmesartan (Sasser *et al.* 2012), further suggesting the distinctive regulation of nNOS splice variants in response to pathophysiological stimulus.

nNOS compartmentation, binding partners and regulation in cardiac and non-cardiac tissues

Other than the essential components that assemble in stoichiometry to form the oxygenase and reductase domains (including co-factors and substrates), growing numbers of proteins are shown to represent 'binding partners' of nNOS. nNOSα and nNOSμ interact with proteins containing PDZ-domain through direct PDZ–PDZ binding or C-terminal–PDZ interactions. These interactions constrain nNOS localization to specialized cell compartments and to specific signal transduction pathways. Despite the importance of such interaction elsewhere, only a few proteins have been confirmed to interact with nNOS in cardiac myocytes and thus much of the current information is derived from findings in the brain or skeletal muscle. To date, nNOS binding proteins in cardiac myocytes are: (i) α-syntrophin; (ii) CAPON, and (iii) Cav3. The first of these, α-syntrophin,

binds to nNOS *via* C-terminal–PDZ domains (in the β-hairpin finger structure region) and docks nNOS to the plasma membrane to form a macromolecular complex with PMCA4b and SCN5a (Ueda *et al.* 2008). CAPON is an adapter/regulator of nNOS in the brain. The C-terminal binding motif of CAPON interacts with the N-terminal PDZ domain of nNOS to activate nNOS (Jaffrey *et al.* 1998). In neurons, CAPON activation of nNOS is important in mediating neuronal excitotoxicity (Zhou & Zhu, 2009) and also facilitates iron uptake and neurotoxicity (Cheah *et al.* 2006). nNOS activation leads to *S*-nitrosylation of dexras1, which binds to benzodiazepine receptor-associated protein (PAP7) to bring dexras1 to divalent metal transporter (DMT1), an iron import channel, and induce iron influx (Cheah *et al.* 2006). Genome-wide analysis in patients with an abnormal QT interval has identified that the polymorphism of a common variant (rs10494366) of the NOS1 regulator, the *NOS1AP* gene (encoding CAPON) is strongly associated with variation in the cardiac QT interval (Arking *et al.* 2006); interestingly, the correlation is more significant in women than in men (Arking *et al.* 2006; Tobin *et al.* 2008). In fact, nNOS activated by CAPON has been implicated in the inhibition of LTCC, subsequently leading to shorter cardiac action potentials (Chang *et al.* 2008). nNOS increases its association with Cav3 in failing myocardium, but the functional relevance of this interaction remains unidentified. Cav3 may inhibit nNOS activity by preventing Ca-CaM binding to nNOS (Garcia-Cardena *et al.* 1997) and attenuate nNOS-derived cellular responses.

A number of nNOS binding proteins are reported in tissues other than myocardium. Representative proteins include: protein inhibitor of nNOS (PIN); a Ca^{2+}-dependent protease, calpain; phosphofructokinase (PFK); various heat shock proteins (HSP90 and HSP70); nitric oxide synthase interacting protein (NOSIP); PSD95; NIDD, and carboxyl-terminal-binding protein (CtBP), etc. PIN physically interacts with nNOS and functions as an endogenous inhibitor of nNOS by destabilizing nNOS dimerization (Jaffrey *et al.* 1996); by binding to CBP, a fusion protein, CBP–PIN also reduces catalytic activity of nNOS without affecting its dimerization (Xia *et al.* 2006). Calpain binding leads to the acceleration of nNOS degradation, which determines the short lifetimes of nNOSα and nNOSμ (~12 min and ~50 min, respectively) (Laine & de Montellano, 1998). PSD95 links nNOS to *N*-methyl-D-aspartate receptor (NMDAR); NMDAR stimulation activates nNOS, which is critical to the postsynaptic activity of nNOS (Brenman *et al.* 1996; Doucet *et al.* 2012). Interestingly, CAPON is shown to compete with PSD-95 and PSD-93 for binding to nNOS, which results in the inactivation and cytosol localization of nNOS (Jaffrey *et al.* 1998). In nNOS-expressing synaptic vesicles, nNOS interacts with PFK and the product of PFK, fructose-1, 6-bisphosphate, may exert a neuroprotective effect (Firestein & Bredt, 1999). Furthermore, nNOS interacts with a transcription factor, CtBP, to target CtBP in cytosol and prevents its localization in the nucleus. By restricting CtBP from interacting with nuclear proteins (i.e. histone deacetylase and transcription factors), nNOS may maintain homeostasis of transcriptional repression and activation (Riefler *et al.* 2001). HSP90 binds to nNOS and enhances its Ca^{2+}–CaM binding and activity (Song *et al.* 2001), and prevents nNOS aggregation and ubiquitination to uphold its stability and location (Corso-Diaz & Krukoff, 2010; Peng *et al.* 2012), all of which mediate protein–protein interaction and receptor–ligand interaction of nNOS to regulate cell function. By contrast, HSP70 promotes nNOS ubiquitination (Peng *et al.* 2012) and plays a contrasting role to HSP90. The identification of nNOS binding proteins and their functional regulations in special compartments are important in pinpointing the roles of nNOS in healthy and diseased myocardium.

Future perspectives: nNOS implications in the diseased heart

Cardiac nNOS regulates basal myocardial contraction and relaxation in the healthy heart. This is an important mechanism that protects against the progression and development of fatal heart diseases. Novel post-translational modifications of nNOS regulation are emerging and enhance our understandings of the important roles played by nNOS in cardiovascular disease. Aspects of nNOS gene regulation in response to pathophysiological stimuli, specific nNOS splice variants, and respective compartment/binding partners are known for various physiological systems, but cardiac-specific knowledge of nNOS and its downstream pathways in different heart diseases is required for the development of effective therapeutic strategies.

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Additional information

Competing interests

None declared.

Author contributions

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