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Cardiac nitric oxide: Emerging role for nNOS in regulating physiological function

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Abstract

Emerging evidence shows that neuronal nitric oxide synthase (nNOS) plays several diverging roles in modulating cardiac function. This review examines the nitric oxide (NO) pathway and the regulatory mechanisms to which nNOS signalling is sensitive. These mechanisms are diverse and include regulation of gene expression, posttranscriptional regulation, protein trafficking, allosteric modulation of nNOS and redox modification to alter NO bioavailability once synthesised. Functionally, alteration in nNOS-NO signalling in the heart may correlate with different cardiac regulatory states. The idea of this being associated with exercise-trained states and myocardial disease is discussed.

Keywords: Neuronal NO-synthase; Contraction; Catecholamines; Autonomic nervous system

Abbreviations: cGMP, cyclic gyanosine monophoshpate; ChAT, choline acetyltransferase; eNOS, endothelial NO synthase; HSP, heat shock protein; IR, immunoreactivity; L-NMMA, L-nitro-monomethylarginine; MI, myocardial infarction; nNOS, neuronal NO synthase; NO, nitric oxide; PDZ, *p*ostsynaptic density protein 95/*d*iscs-large/*z*ona occlusens-1; PIN, protein inhibitor of nNOS; PSD-95, postsynaptic density-95; SOD, superoxide dismutase.

Contents

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1. Introduction

The role of nitric oxide (NO) in the control of cardiac function has been studied extensively over the last 10 years (comprehensive recent reviews by Hare et al., 1995; Paton et al., 2002; Hare, 2003; Massion et al., 2003; Sears et al., 2004), after a fervent drive was established for research in this field by the dominant role NO is now known to play in vascular regulation. Although this has yielded numerous interesting and important discoveries, it has not quelled the mystical nature of NO, nor the huge diversity of (often conflicting) experimental results it appears to elicit. Part of the explanation for this may come from the wide range of techniques and experimental preparations currently being used for studying NO biology. Novel findings have revealed that such variability may influence the physiological actions of NO since it is involved in numerous regulatory processes sensitive to different biological conditions. In addition, several mechanisms are involved in regulation of NO generation and bioavailability in the heart making interpretation of its physiological role often contextual. One of the most important advances in understanding of this came with the discovery of spatially localised neuronal NO synthase (nNOS or NOS1) enzyme expression in cardiac tissues, which is functionally and spatially discreet from the endothelial NOS (eNOS or NOS3) isoform found in vascular and endocardial endothelium. Unlike eNOS, the function and regulation of nNOS-derived NO in the heart have not been well characterised, although it is thought to be subject to substantially more transcriptional diversity.

The ability of nNOS to manipulate cardiac function is of significant future interest, since many of its documented roles involve modulating autonomic balance and calcium signalling within the heart towards more favourable, anti-arrhythmic states. This article reviews recent advances in our understanding of the role of nNOS in cardiac function with specific focus on the regulation of gene expression, protein function and the functional correlates of altered NO bioavailability.

2. Neuronal nitric oxide synthase: General structure and function

2.1. Properties of nitric oxide

NO is a member of the labile radical entities known as reactive oxygen species (ROS) and contains 1 nitrogen atom covalently bonded to an oxygen atom with 1 unpaired electron. It is particularly reactive with oxygen and haeme-iron containing groups which reduce NO to more stable nitrate compounds (for review, see Ignarro, 1989). For this reason the bioavailability of NO in certain tissues (notably blood-rich in haemoglobin and muscle-rich in myoglobin; Ignarro et al., 1993) is extremely low and the biological actions are restricted temporally and spatially close to its site of synthesis. Paradoxically, NO is also lipid-soluble, making it highly membrane permeant (Subczynski et al., 1996); therefore, many of its most well-described actions involve its diffusion between cells to act as a paracrine-signalling molecule. This makes quantifying NO with respect to the functional correlates of NO signalling a difficult task, and the term NO "bioavailability" is often used to quantify the functional potential for endogenously synthesised NO in any one tissue at any one time. These somewhat contradictory properties of NO may, however, be appropriate to its functional roles within the heart, where discreet NO-producing microdomains are flanked by myoglobin-rich, NO-scavenging ones, thus making its biological action more site-specific. In the myoglobin knockout mouse, the biological actions of endogenously synthesised NO in the heart are augmented and fundamentally altered (Flogel et al., 2001). In contrast, other cardiac tissues, for example, coronary vascular endothelium, depend on intercellular NO diffusion to local smooth muscle tissue of the tunica media in order to carry out its biological action (vasodilatation). After synthesis, NO has a number of different fates. Many of the biological actions described in the literature so far have involved the interaction of NO with soluble guanylyl cyclase and the production of cyclic gyanosine monophoshpate (cGMP). However, a number of cGMP-independent roles have also been described (for review, see Smith et al., 1996). These include the nitrosylation of the cardiac ryanodine receptor (RyR), thereby enhancing calcium spark frequency (Petroff et al., 2001), and the generation of peroxynitrite (from reaction with superoxide, which may also be generated by uncoupled NOS activity; Witteveen et al., 1999).

2.2. Neuronal nitric oxide synthase gene and protein structure

The human nNOS gene maps to the q14-qter region of chromosome 12 (Kishimoto et al., 1992); with homologous genes on chromosome 5 of the mouse genome (Lee et al., 1995) and chromosome 12 in the rat (Deng et al., 1995).

The locus of the human nNOS gene is scattered over 200 kb and the mRNA transcript found in neuronal tissue is encoded by 29 exons (1434 AAs; 160 kDa). Analysis of intron-exon splice junctions predicted that the open-reading frame is encoded by 28 exons, with translation initiation and termination in exon 2 and exon 29, respectively (see Fig. 1). However, genotypic analyses have demonstrated multiple alleles, mainly resulting from transcription using alternative promoters. These are also subject to posttranscriptional modification including cassette exon deletion or insertion to give the well-described splice variants nNOSα (155 kDa), nNOSβ (136 kDa), nNOSγ (125 kDa), and nNOS-2 (105 kDa, although numerous other variants have been found (see Section 4.2). nNOSα is the most commonly expressed variant in striated muscle and neural tissue, although mature skeletal muscle (Silvagno et al., 1996) and human and rat penis and urethra (Magee et al., 1996) express a variant nNOSμ (164 kDa) following exon insertion between the calmodulin and flavin-binding domains. nNOSβ and nNOSγ are derived from transcripts following deletion of exon 2, which encodes the *p*ostsynaptic density protein 95/*d*iscs-large/*z*ona occlusens-1 (PDZ) binding domain which precludes this variant from associating with synaptic membranes (Brenman et al., 1996). Furthermore, of all of these splice variants, additional variation in size exists owing to different numbers of CA/TG dinucleotide repeats in the 5′ flanking region (Hall et al., 1994). It is thought that variation of this kind allows for differences in basal promoter activity (Takahashi et al., 1997).

2.3. Nitric oxide-protein interaction

The rate of synthesis of NO by nNOS is 6 times that of eNOS (Nishida & Ortiz de Montellano, 1998). However, the synthesis of NO from either isoform per se depends upon the individual protein variant, its allosteric regulators and availability of its substrates, L-arginine and oxygen, and cofactors NADPH, calmodulin, flavin nucleotide reductases (FAD and FMN), haeme, and tetrahydrobiopterin (BH_4).

The biological actions of NO described so far mainly relate to its interaction with other ROS or cellular protein domains, although redox modulation of potentially any targets are possible (for review, see Alderton et al., 2001). In the heart, the functional role of NO is likely to depend largely on the spatial proximity of the target to the active nNOS enzyme and the oxidative stress of the microenvironment. Redox regulation of sulfhydryl protein domains of associated cellular structures is 1 mechanism through which nNOS is thought to act, this mechanism being particularly relevant to regulation of calcium cycling through the RyR (for review,

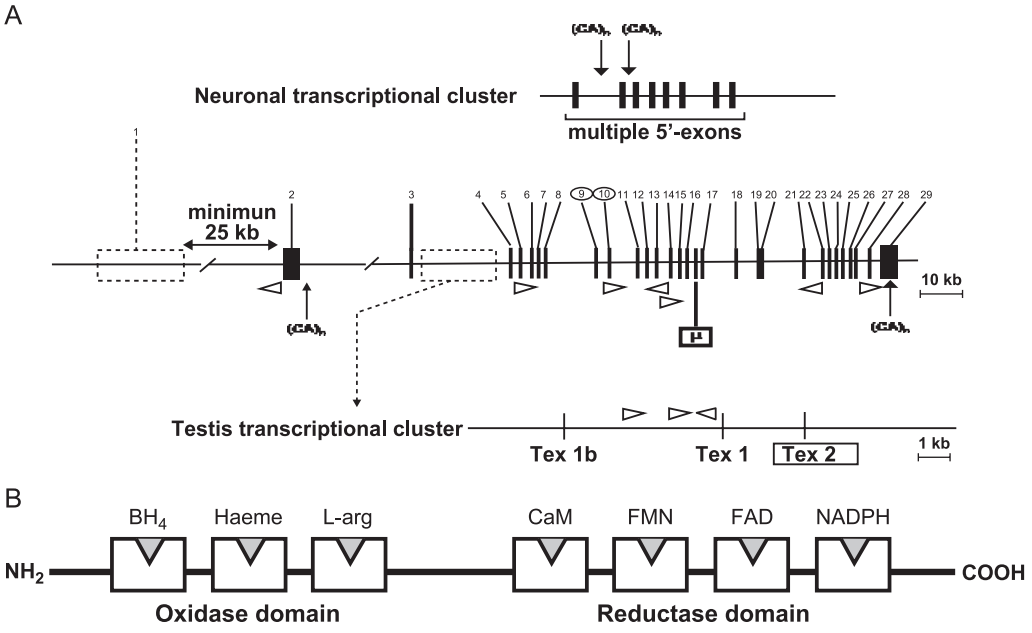

Fig. 1. (**A**) Genomic organisation of the human neuronal nitric oxide synthase (nNOS; adapted from Forstermann et al., 1998). The nucleotide sequence corresponding to the major neuronal mRNA transcript is encoded by 29 exons. Many exons are of relatively small size, ranging from 59 to 266 bp. Two described transcriptional clusters (neuronal and testis specific) are shown above and below the genomic map. Due to the presence of multiple promoters and transcription start sites, different first exons (5′-exons) can be used. All first exons of the neuronal cluster seem to splice to the common exon 2; therefore, 1 unique translation product corresponding to the full-length nNOS protein is likely to be assembled. Transcription from the testis-specific cluster, located between exon 3 and exon 4, gives rise to mRNA transcripts encoding for NH_2-truncated enzymes. Possible cassette exon deletions of exon 10, or exons 9 and 10, are circled. Reported cassette exon insertions in the full-length nNOS are boxed: Tex 2 from the testis transcriptional cluster, and the μ-nNOS insert located between exons 16 and 17 of the human nNOS. Locations for repetitive elements are indicated; the human gene contains 3 sets of dinucleotide CA repeats and 1 (CG)n island. Members of the Alu repetitive sequences, indicated by triangles, are also found scattered at several locations along the human nNOS gene. (**B**) Diagram representing 1 monomer and the functional organisation of NOS enzyme protein. Electron transfer occurs from the carboxy-terminal at the reductase domain, starting from NAPDH to the flavin molecules FAD and FMN; a process triggered by calcium binding to calmodulin (CaM).

see Salama et al., 2000). In addition, such proteins are modulated by *S*-nitrosylation. At greater oxidative stress, protein domains may be more or less liable to undergo redox reactions with NO. In addition, NOS synthesises proportionally less NO and other ROS are formed including nitroxyl (NO^-), peroxynitrite ($ONOO^-$), and nitrosothiols (Schmidt et al., 1996). Formation of NO by nNOS (which can be augmented by superoxide dismutase [SOD] and low oxidative stress) leads to preferential activation of sGC and cGMP formation.

3. Neuronal nitric oxide synthase localisation and putative roles

3.1. Cardiomyocytes

Both eNOS and nNOS are expressed in cardiomyocytes, although the subcellular organisation of these enzymes is discreet. Furthermore, this localisation may be complex in as such as the trafficking NOS protein within the cardiomyocyte may interact with the functional roles of the enzyme. Targeting of nNOS to appropriate subcellular microdomains is mediated by interactions with its PDZ domain and scaffold adaptor proteins. In skeletal muscle, nNOS forms a complex with dystrophin and α-syntrophin, with binding occurring via the PDZ domain of α-syntrophin (Brenman et al., 1995). Although these scaffold proteins are also found in cardiac muscle, their relationship with cardiac nNOS protein is not known. In addition, nNOS binds to caveolin isoforms normally found in membrane caveolae (see Fig. 2), which act as scaffold proteins and regulate enzyme activity by competing with calcium and inhibiting NO biosynthesis (Michel et al., 1997). In skeletal muscle, nNOS associated with caveolin-3 has been identified (Venema et al., 1997), whereas it is associated with caveolin-1 in the smooth muscle of the lower oesophageal sphincter (Daniel et al., 2001) and in vascular smooth muscle (Cheah et al., 2002). However, although the association between eNOS and caveolin-3 is well known in the heart, this relationship is not well

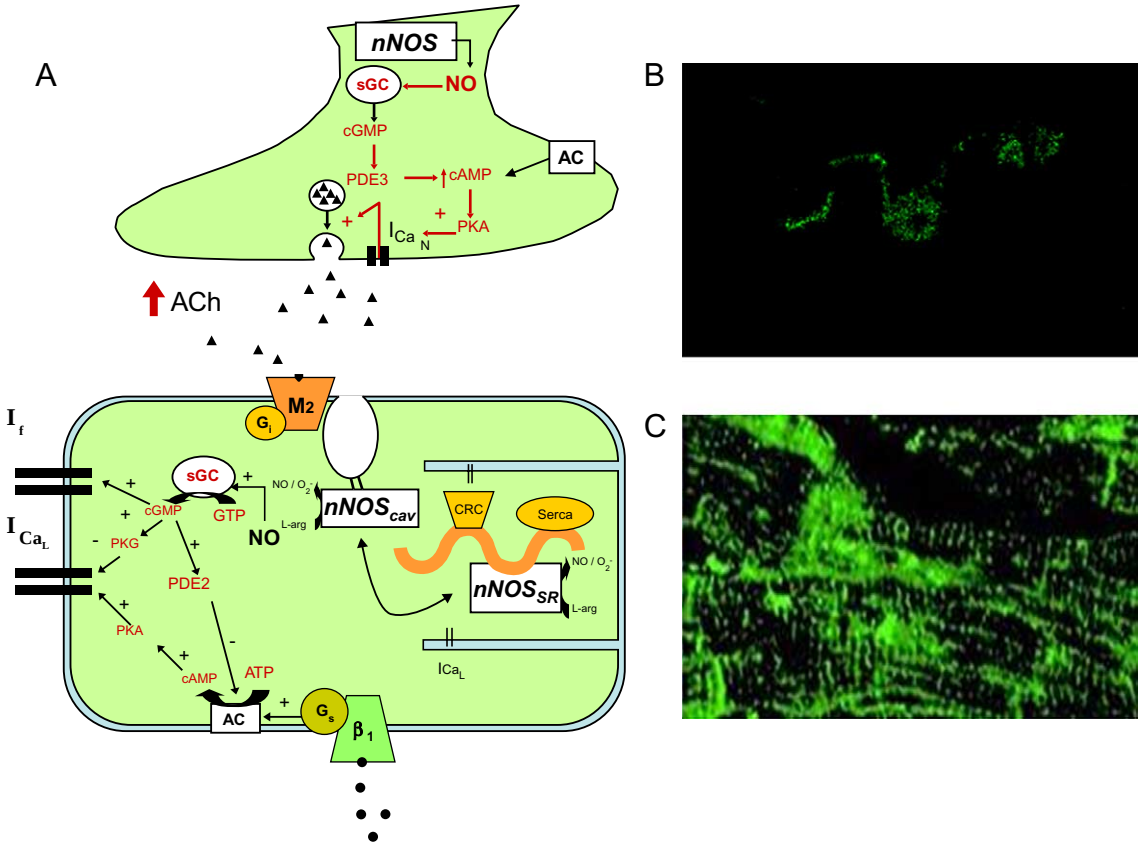

Fig. 2. (**A**) Schematic diagram illustrating putative roles of nNOS in cardiac cholinergic neurones and myocytes. In neurones, nNOS synthesizes NO which acts to increase cyclic guanosine monophosphate (cGMP) via souble guanylyl cyclase (GC). This may inhibit PDE3 to increase cAMP- and PKA-dependent phosphorylation of N-type calcium channels (I_{CaN}), and facilitate acetylcholine (ACh) release and vagal bradycardia modified from Paton et al., 2002. In myocytes, nNOS may reside in microdomains associated with the sarcoplasmic reticulum (SR) and caveolae (CAV). At the SR, NO may modulate calcium cycling by attenuating calcium release at the calcium release channel (CRC) or by modulating sarcoplasmic reticulum calcium-ATPase pump (SERCA) activity. Within caveolae, NO may inhibit calcium influx by cGMP-dependent inhibition of the L-type calcium channel (I_{CaL}). (**B**) Immunolocalisation of nNOS in intrinsic cardiac parasympathetic neurone (adapted from Choate et al., 2001). (**C**) Immunolocalisation of nNOS in ventricular myocyte (adapted from Damy et al., 2003).

established for caveolins and nNOS in the normal (non-diseased) heart. In contrast, nNOS has been immunolocalised within sarcoplasmic reticulum (SR) vesicles in normal rabbit LV myocytes (Xu et al., 1999) where the scaffold proteins responsible for this interaction are not known.

The significance of endogenous NO signalling in cardiomyocytes was originally studied through the effect of exogenously applied NO donors like sodium nitroprusside (SNP). In general, this is not an ideal method for eliciting the physiological role of either NOS isoform since the temporal and spatial characteristics of endogenous NO release are not replicated by NO donors. However, the broadly repeatable effect of most NO donors on myocardial inotropy and chronotropy is biphasic (Kojda et al., 1996; Mohan et al., 1996; Wegener et al., 2002; see Table 1). Whilst low doses of SNP increase cardiac contractility (Mohan et al., 1996) and rate (Musialek et al., 1997), higher doses have a negative inotropic and chronotropic effect. A slightly more reliable means of establishing the effects of endogenous NO, but by no means ideal, is through the use of NOS inhibitors, some of which are reported to be moderately isoform selective, or by using eNOS/nNOS gene knockout. Although some evidence suggests that endogenously synthesised NO has a mild positive inotropic effect in cardiomyocytes by using NOS inhibitors (Kojda et al., 1997; Cotton et al., 2001), this is at odds with the effect of eNOS gene knockout (Gyurko et al., 2000; Godecke et al., 2001). Furthermore, suggestions that eNOS knockout mice may have lower basal heart rates independent of the baroreflex-mediated response to hypertension in these mice (Sumeray et al., 2000) have not been substantiated in later reports (Scherrer-Crosbie et al., 2001). Likewise, evidence supporting a role for nNOS in the modulation of cardiac inotropy or chronotropy is not consistent (Sumeray et al., 2000; Barouch et al., 2002), although a recent study has shown that basal calcium current density is reduced in nNOS knockout ventricular myocytes (Sears et al., 2003).

In general, the effects of NO on cardiomyocyte function are more substantial where there are autonomic influences at the time of experimentation, and this may give a more physiological representation of how it modulates cardiac function. Despite the fact that early evidence appeared to support a role for cardiac NO in the autocrine regulation of cholinergic signalling, more recent work on this mechanism has failed to find many of the originally documented effects. The early work showed that cholinergic inhibition of cardiac chronotropy could be blocked by endogenous NOS inhibition or NO scavengers in rat neonatal myocytes (Balligand

Table 1
Effects of NO manipulation on basal and stimulated cardiac function

Preparation	Intervention	Effect (basal)	Effect (cholinergic response)	Effect (β-adrenergic response)
LV myocytes	NOS inhibition	No effect (Balligand et al., 1993; Harding et al., 1998)	Decreased chronotropic effect (Balligand et al., 1993)	Increased inotropic effect (Balligand et al., 1993)
		No effect (Vandecasteele et al., 1999)	No effect (Vandecasteele et al., 1999)	No effect (Vandecasteele et al., 1999)
	nNOS knockout	Increased inotropy (Sears et al., 2003; Ashley et al., 2002)	–	Increased inotropic effect (iso, 2 nmol/L) (Ashley et al., 2002)
		No effect (Barouch et al., 2002)	–	Decreased inotropic effect (iso, 1 μmol/L) (Barouch et al., 2002)
		Decreased force-frequency response (Khan et al., 2003)		
Isolated-perfused heart	NOS inhibition	Negative inotropy (Kojda et al., 1997; Brunner et al., 2001)	No effect (Godecke et al., 2001)	Increased inotropic effect (Sterin-Borda et al., 1998)
	nNOS knockout	–	–	–
Innervated in-vitro heart	NOS inhibition	No effect (Choate et al., 2001; Danson et al., 2002)	Decreased chronotropic effect (presynaptic) (Choate et al., 2001; Danson et al., 2002)	Increased chronotropic effect (presynaptic) (Mohan et al., 2000)
	nNOS knockout	Increased HR (Choate et al., 2001)	Decreased chronotropic effect (presynaptic) (Choate et al., 2001)	–
In-vivo	NOS inhibition	No effect (Hare et al., 1995)	Decreased chronotropic effect (Chowdhary et al., 2000)	Increased chronotropic effect (Takita et al., 1998; Keaney et al., 1996)
	nNOS knockout	Increased HR (Jumrussirikul et al., 1998)	Decreased HR response to atropine (Jumrussirikul et al., 1998)	No effect on response to propanolol (Jumrussirikul et al., 1998)

et al., 1993). This concept was developed further by Han and coworkers who demonstrated that NOS inhibitors (Han et al., 1994) or eNOS gene knockout (Han et al., 1998) prevented cholinergic inhibition of the L-type inward calcium current (I_{CaL}) in adrenergically prestimulated rabbit sinoatrial node cells. They characterised this pathway as involving cGMP-dependent stimulation of phosphodiesterase 2 (PDE2), which reduces cAMP- and protein kinase A (PKA)-dependent stimulation of I_{CaL}. This has led to speculation that NO plays an obligatory role in the autonomic control of heart rate (Han et al., 1994), although others fail to confirm this idea (Vandecasteele et al., 1998), and have shown that eNOS knockout mice exhibit normal autonomic activation of I_{CaL} (Vandecasteele et al., 1999; Belevych et al., 2000; Godecke et al., 2001). Since the expression of other NOS isoforms was unaltered, these results provide compelling evidence against a significant role for eNOS-dependent NO generation in the autonomic control of cardiac function; although there are distinct methodological differences between both groups (for review, see Herring et al., 2002).

For example, the use of knockout animals is complicated by the difference in age of the animals used. Whilst Han et al. used mice at 2 days to 2 months old, Vandecasteele et al. and Godecke et al. used mice at 3–6 months and Belevych and Harvey (2000) used mice at 2–4 months old. As a consequence of eNOS gene knockout in the vasculature, these animals develop hypertension secondary to a high total peripheral resistance. In addition to compensatory changes that result directly from removal of the eNOS gene on the cellular level, changes may also result in response to hypertension-induced hypertrophy in older animals that are not present in wild types. Another factor to confound the interpretation of results is that both NOS activity and calcium channel behaviour are exquisitely sensitive to temperature, and some studies that have failed to find a role for NO were carried out at room temperature (Vandecasteele et al., 1999) rather than 37 °C (Han et al., 1994, 1998). Furthermore, basal NOS activity or the presence of the NOS enzyme is rarely assessed. eNOS is inhomogeneously expressed across the ventricular wall. It is also possible that there may be a switching from NO to adenylate cyclase-dependent regulation of I_{CaL} during development (Ji et al., 1999), and so the age of the animal and cell isolation technique must be controlled. The effect of NOS inhibition is also likely to be critically dependent on the level of prior adrenergic stimulation and how high cAMP levels have been raised. When measuring I_{CaL}, it is difficult to carry out full dose-response curves during short patch-clamping protocols, and studies use many different concentrations of isoprenaline (rather than the physiological agonist noradrenaline).

The effects of NO on adrenergic signalling in cardiomyocytes using the various methodological approaches has also revealed discrepant results. Although many reports claim using pharmacological inhibition of NOS augments inotropic responsiveness to β-agonists at the cellular (Goldberg & Haddox, 1977), tissue (Sterin-Borda et al., 1998), and whole animal (Keaney et al., 1996) level whilst exogenous NO donors exert the opposite effect (Choate & Paterson, 1999), distinguishing the contributory roles of eNOS and nNOS has been difficult. With respect to eNOS, although $eNOS^{-/-}$ mice in vivo (Gyurko et al., 2000; Barouch et al., 2002) or Langendorff-perfused hearts (Gyurko et al., 2000; Godecke et al., 2001) demonstrate augmented responses to β-agonists, this effect has for the most part not been replicated in isolated $eNOS^{-/-}$ myocytes (Han et al., 1998; Vandecasteele et al., 1999; Belevych et al., 2000; Godecke et al., 2001) except where doses of β-agonists used exceed 10^{-7} M (Barouch et al., 2002), suggesting that a significant contribution comes from endothelial endocardium (Pinsky et al., 1997). However, where the targeted expression of eNOS in cardiomyocytes is amplified by transgenic overexpression (Brunner et al., 2001) or other means (Danson et al., 2002) β−adrenergic contractility is significantly attenuated, suggesting it may play some inhibitory role.

The role of nNOS, however, is more controversial. SR-associated nNOS (Xu et al., 1999) may modulate Ca^{2+} signalling in ventricular myocytes. It is suggested that nNOS may exert a tonic inhibitory effect in the basal regulation of the L-type Ca^{2+} current and intracellular calcium transients (Sears et al., 2003), although this role has been disputed (Khan et al., 2003). Contradictory results have also been seen using the nNOS knockout mouse, with some studies reporting an increase in responsiveness to low doses of β-agonists (Ashley et al., 2002) whilst others report the opposite effect with higher doses (Barouch et al., 2002).

3.2. Intrinsic neurones

Neural tissue supplying the heart can broadly be divided into efferent sympathetic neurones derived predominantly from the stellate ganglia, parasympathetic neurones from the vagus nerve which exclusively synapse within intrinsic cholinergic ganglia, from which postganglionic neurones are derived; and afferent neurones carrying sensory information back to the CNS through various neural pathways. All intrinsic cardiac neurones innervate numerous sites within the heart, and most are thought to be modulated to some extent by NO. However, the majority of intrinsic cardiac neurones that contain nNOS protein are likely to be parasympathetic because they show colocalisation of immunoreactivity (IR) for choline acetyltransferase (ChAT), an enzyme involved in the synthesis of the parasympathetic neurotransmitter acetylcholine, and exist within intrinsic ganglia (Choate et al., 2001). Only a small proportion of intrinsic ganglion cells contain nNOS; in the guinea-pig atria 5% of the ChAT-immunoreactive neurones were also nNOS-IR (Mawe et al., 1996). Similarly, nNOS was found in only in 4% of intracardiac neurones in the rat (Klimaschewski et al.,

1992). More recently, nNOS was colocalised in 16% of ChAT neurones within cardiac ganglia in the mouse atria (Danson & Paterson, 2003). The variability between these estimates may reflect species differences in the proportion of nNOS-IR intrinsic neurones, and that the mouse may utilise nNOS-derived NO to a greater extent as a neuromodulator compared to other rodents.

Although the trafficking of nNOS was first described in central neurones where it is either soluble (Hecker et al., 1994) or synaptically localised to both glutamate receptor-containing central synapses and the mammalian motor endplate (Brenman & Bredt, 1997), this has not been specifically investigated in autonomic ganglia. Protein in neural tissue, however, is thought to be predominantly nNOSα and is associated with the PDZ binding domains distributed throughout the neuronal synaptic terminal. Localisation of nNOS also broadly follows the distribution of NMDA receptor calcium channels, which contains a PDZ-binding domain (Baranano & Snyder, 2001); and transcription of nNOS is potently regulated by free calcium (Sasaki et al., 2000).

The effects of NO on peripheral neurones may also be characterised according to neural pathways. The effects on the parasympathetic nervous system are extrapolated from experiments by vagotomising animals in vivo, or isolating the heart with intact vagal innervation for study in vitro. Peripherally, inhibition of nNOS causes a dramatic reduction in the vagally mediated bradycardia in the ferret and guinea pig in vivo (Conlon & Kidd, 1999), and a modest effect in the dog (Elvan et al., 1997) whereas others report no significant effect in the guinea pig in vitro (Sears et al., 1998) or rabbit in vivo (Liu et al., 1996; Sears et al., 1998). In humans, NO donors can increase the high frequency component of heart rate variability, an index of cardiac vagal tone (Chowdhary & Townend, 1999). Additionally, NO donors and the cGMP analogue 8-bromo-cGMP enhance the heart rate response to vagal nerve stimulation in rabbits and guinea pigs (Sears et al., 1999). However, the bradycardic response to carbamylcholine (the stable analogue of acetylcholine) is unaffected by NOS or guanylate cyclase inhibitors, consistent with the idea that NO is a presynaptic modulator of vagal neurotransmission (Sears et al., 1999; Herring et al., 2000). Furthermore, NO or cGMP can cause an increase in acetylcholine release during field stimulation of the right atrium (Herring & Paterson, 2001). This is thought to result from increased cGMP-mediated inhibition of PDE3, leading to increased PKA-dependent phosphorylation of N-type calcium channels and calcium-mediated exocytosis. The likely source of NO is from nNOS, since conscious nNOS knockout mice have a higher baseline heart rate and blunted HR response to atropine (Jumrussirikul et al., 1998). Moreover, isolated atria from $nNOS^{-/-}$ demonstrate attenuated responses to peripheral vagal nerve stimulation compared to wild-type controls (Choate et al., 2001).

Effects of NO on peripheral sympathetic nerve terminals have also been documented, but the magnitude of these effects are more difficult to determine in isolation since it is more widely accepted that NO from numerous sources modulates β-adrenergic responsiveness in cardiomyocytes. Some evidence exists nonetheless that NO acts presynaptically to inhibit cardiac sympathetic neurotransmission, both in vivo and in vitro. NOS inhibition enhances overflow of noradrenaline during cardiac sympathetic nerve stimulation in the isolated perfused rat heart (Schwarz et al., 1995), and increases the inotropic effect of stellate ganglion stimulation (Takita et al., 1998) and β-agonists (Elvan et al., 1997) in vagotomised dogs. Inhibition of NOS also increases chronotropic responsiveness to stimulation of the stellate ganglion, both in rabbits in vivo (Sears et al., 1998) and guinea pigs in vitro (Choate & Paterson, 1999), although chronotropic responsiveness to β-agonists is unaffected. It is unclear therefore whether endogenous NO may exert differential effects on cardiac contraction and rate under conditions of β-adrenergic stimulation; acting prejunctionally to inhibit release of noradrenaline from the sympathetic varicosity, but also postjunctionally to inhibit the inotropic responsiveness of target myocytes.

4. Neuronal nitric oxide synthase-nitric oxide regulation

In recent years, the number of regulatory mechanisms linked to the control nNOS-NO biosynthesis has multiplied, and this continues to grow rapidly. In respect of the central dogma of gene expression, posttranscriptional and post-translational modification, protein phosphorylation, redox modulation and protein-protein interactions, nNOS appears to be exquisitely sensitive at most steps.

4.1. Regulation of gene expression

Most of the evidence surrounding nNOS gene expression regulation is biased towards the mechanisms discovered in neuronal tissue, since this is where the protein was first discovered. nNOS mRNA up-regulation seems to represent a general response in neuronal cells to stress induced by a large array of physical, chemical and biological agents such as heat, light exposure, electrical stimulation, and allergic substances (for review, see Forstermann et al., 1998). Furthermore, nNOS is often associated with coinduction of transcriptional factors such as c-*Jun* (Wang et al., 2002). Also, several in vivo studies in rats suggest a time-dependent increase in nNOS mRNA after hypoxia (e.g., Guo et al., 1997). This up-regulation may be due to 2 distinct mechanisms: a general cellular stress response or a direct activation of the nNOS gene transcription through binding of hypoxia-induced factors to specific *cis*-acting elements, as seen in erythropoietin and several other hypoxia-induced genes (Kvietikova et al., 1995). Putative "BACGTSSK" binding sites for the hypoxia-inducible factor-1 (HIF-1) can be

detected along the nNOS genomic sequence, but the functional significance of these motifs has not yet been determined. Furthermore, little is known to correlate the various alternative promoter sequences with tissue or stimulus-specific responses.

Interestingly, levels of nNOS protein within homogenised cardiac atria from guinea pigs have been shown to decrease after exposure of the animals to intermittent hypoxia for 21 days (Mohan et al., 2001b). However, this response is accompanied by a decrease in stress protein levels (including heat shock protein 70 [HSP 70] and HSP 90; Mohan et al., 2001a), suggesting that cell stress responses may in some contexts dictate hypoxic responses in the control of nNOS protein expression in cardiac tissue.

4.2. Posttranscriptional modification

Cassette insertion or deletions to the nNOS transcript are common and are thought to modify nNOS activity and function. However, how this correlates with cardiac function has not been the focus of any investigation to date. It is clear that in skeletal muscle, a change in the molecular weight of nNOS (conversion to nNOSμ) underpins an important part of development (Silvagno et al., 1996). Similar changes in nNOS expression are thought to occur during maturation of the nervous system (Bredt & Snyder, 1994) and lung (North et al., 1994). In the heart, various splice variants have been detected, albeit in different reports and using different techniques. Silvagno et al. (1996), who first described nNOSμ, used purification of nNOS protein and generation of a novel nNOSμ antiserum. Various other commercially available antibodies have been utilised to detect nNOS protein within cardiac tissue of molecular weights of 120 kDa (Herring et al., 2000), 155 kDa (Moreno et al., 2002; Danson & Paterson, 2003), 160 kDa (Xu et al., 1999; Takimoto et al., 2000) and 164 kDa (Lin et al., 1998). However, some doubt exists over the specificity of many of current commercially available antibodies for nNOS (Coers et al., 1998), and this has made interpretation of nNOS gene regulation via Western blot or immunologically based imaging techniques difficult.

Changes in the molecular weight of nNOS protein in the heart with stress or age remain a possibility. In both cardiac atria (Herring et al., 2000) and ventricular myocytes (Piech et al., 2003) increases in nNOS have been seen as a consequence of age. In the study concerning atria in particular, a different molecular weight of nNOS was observed (Herring et al., 2000). However, detailed understanding of how this correlates with function is still currently at a rudimentary stage. It is likely, however, since posttranscriptional modification impinges on both trafficking and enzymatic function of the protein end-product, that such posttranscriptional changes to nNOS protein result in dramatically different biological roles being taken on.

4.3. Posttranslation modification

Unlike eNOS, the process of posttranslational modification of nNOS with respect to protein trafficking to different cell domains has not been fully elucidated. This may be an important area of future interest to many investigators since the localisation of NOS protein appears to play a defining role in its regulatory function in cardiomyocytes (Barouch et al., 2002). Myristolation is essential for association of eNOS to the golgi complex and thereby its trafficking to the sarcolemma, whilst palmitoylation, on the other hand, allows for targeting to caveolae (Shaul et al., 1996). Although no evidence exists to suggest that nNOS is expressed in cardiomyocyte membrane caveolae in normal hearts, this has been documented in failing human LV myocytes (Damy et al., 2004), an effect associated with the maintenance of a normal dP/dt_{max} value and ventricular relaxation in infarcted mouse hearts (Damy et al., 2003). How this translocation process takes place, however, is not known. Studies to date suggest that trafficking of nNOS is mediated predominantly by modification of the binding domain protein, rather than nNOS itself. In neuronal cells, nNOS is targeted to the NMDA receptor via the competitive interaction with the PDZ domains of the neuronal scaffold proteins, postsynaptic density-95 (PSD-95; Brenman et al., 1996) and CAPON (Jaffrey et al., 1998). The interaction with PSD-95 in particular specifically configures nNOS for allosteric regulation by Serine 847 phoshorylation (Watanabe et al., 2003). Numerous other proteins with PDZ domains are now known (for review, see Kone et al., 2003), although the regulatory roles of each interaction may be tissue-specific and that used in the heart is not known. In addition, PSD-95 is known to be capable of associating with membrane caveolae through an unknown mechanism in brain tissue (Perez & Bredt, 1998), thereby potentially exposing nNOS to inhibitory regulation by caveolins.

4.4. Protein phosphorylation

Regulation of nNOS by phosphorylation is now thought to be an important mechanism by which enzyme activity is negatively regulated (Dinerman et al., 1994). Only serine phosphorylation sites have been characterised on the nNOS protein thus far, as opposed to serine, threonine, and tyrosine sites identified on eNOS protein, most of which stimulate enzyme activity (for a review, see Fleming & Busse, 2003). Extensive work has shown that activation of calcium/calmodulin (Cal-CaM)-dependent protein kinases I (Ser 741; Song et al., 2004) and II (Ser 847; Hayashi et al., 1999) can induce serine phosphorylation of nNOS to decrease enzyme activity, a process catalysed by PSD-95/PDZ association (Watanabe et al., 2003). The functional significance of this pathway is not understood, although some evidence suggests that inhibition of nNOS by

phosphorylation during brain ischaemia may limit the damage caused by NO (Osuka et al., 2002; Yan et al., 2004).

4.5. Redox modulation

The activity of nNOS protein appears to be highly sensitive to changes in oxidative stress. This is most convincingly demonstrated by the dramatic and repeatable effects of SOD or SOD mimetics on NO synthesis (Hobbs et al., 1994; Schmidt et al., 1996). Whether all of these effects are brought about by redox modulation of nNOS by free radicals or directly or indirectly by SOD itself is not clear. However, H_20_2, NO^-, and $ONOO^-$, which are all potential products of NOS catalysis, and NO itself all autoinhibit purified NOS enzyme with different potencies (Kotsonis et al., 1999).

Fluctuations in tissue oxidative stress are dependent on numerous factors. Physiologically, the greatest quantitative changes take place during exercise (Salo et al., 1991). Similar increases have been observed with age or myocardial infarction (MI; Ferrari et al., 1998), and prolonged increases in superoxide levels also develop in the LV in hypertensive rats (Piech et al., 2003). These effects are assumed to decrease NO bioavailabilty and may put stress on the other regulatory mechanisms controlling NOS activity to maintain NO levels.

4.6. Protein-protein interactions

Allosteric regulation of NOS isoforms represents an important mechanism by which enzyme activity is subject to rapid modulation and is therefore relevant to acute myocardial changes such as MI or exercise. The regulatory roles of the 2 scaffold proteins in neurones PSD-95 and CAPON have been discussed earlier. Two other well-described protein modulators also exist in cardiomyocytes: caveolin-3 (inhibitory) and HSP 90 (stimulatory) are both thought to regulate nNOS catalysis through altering its activation by Cal-CaM. However, debate exists over whether either mechanism operates entirely as a simple allosteric regulator or whether interaction of nNOS with caveolins or HSP 90 acts as a scaffold for the recruitment of other regulatory mechanisms including kinases and phosphatases. In particular, changes in the expression of either caveolin-3 (Hare et al., 2000; Bendall et al., 2004) or HSP 90 (Piech et al., 2003) do not necessarily produce expected effects on NOS protein activity in cardiomyocytes, given the acute effects of either in vitro, suggesting that the regulatory roles may be more complex.

The relationship between caveolin proteins and NOS was first established by early evidence demonstrating that the addition of calmodulin disrupted the heteromeric complex formed between eNOS and caveolin-3 (a scaffold protein found in membrane-caveolae) in a calcium-dependent fashion, leading to the speculation that caveolin-3 interfered with activation of eNOS through calcium (Michel et al., 1997). It is now known that eNOS activity at any one time is essentially a function of the available calcium relative to the background antagonistic action of caveolin-3 in cardiomyocytes. This relationship is known to exist for nNOS and caveolins in vitro (Sato et al., 2004) and in skeletal muscle (Venema et al., 1997); however, the specific regulatory roles played in cardiomyocytes is not known. It has recently been shown that failing rat LV myocytes express increased levels of both caveolin-3 and membrane-associated nNOS (Bendall et al., 2004), and nNOS coimmunoprecipitation with caveolin-3 is increased in LV myocytes following coronary infarction (Damy et al., 2003). The authors of this study speculate that rather than having an inhibitory effect, increased membrane compartmentalisation of nNOS by caveolin-3 may lead to augmented nNOS-NO bioavailability by bringing the enzyme into close proximity to substrates and calcium, as others have suggested with respect to eNOS in failing canine myocardium (Hare et al., 2000). In both cases NOS activity was augmented as measured by the effect of the nNOS inhibitor, L-VNIO on L-citrulline formation (Bendall et al., 2004) or the nonselective constitutive NOS inhibitor, L-nitro-monomethylarginine (L-NMMA) on contractility (Hare et al., 2000) on failing heart tissue with respect to sham controls.

In contrast, HSP 90 is known to acutely enhance the affinity of nNOS for CaM in vitro (Song et al., 2001) and, interestingly, decrease the synthesis of superoxide by nNOS in favour of NO synthesis (Song et al., 2002). The precise effects of HSP 90 in vivo are not clear since this has only been studied in cultured cells transfected with nNOS and using geldanamycin as a specific HSP 90 inhibitor (Bender et al., 1999). Unfortunately, geldanamycin is also known to act as a tyrosine kinase inhibitor and suppresses oncogene expression (Mimnaugh et al., 1996). Coimmunoprecipitation of HSP 90 and nNOS is increased in LV myocytes from failing human hearts (Damy et al., 2004) and rat hearts after MI (Damy et al., 2003). This is associated with augmented nNOS activity, although the causal relationship of HSP 90 in producing this effect has not been established. Furthermore, increased expression of both nNOS and HSP 90 in LV myocytes from hypertensive rats has no effect on NO synthesis (Piech et al., 2003).

An endogenous protein inhibitor of nNOS (PIN; Jaffrey & Snyder, 1996) has also been identified using a yeast 2 hybrid screen. PIN acts as a specific inhibitor of nNOS by destabilising dimerisation of nNOS protein, essential for functional activity. Although PIN has been used to explain functional differences in nNOS activity in the brain (Greenwood et al., 1997), its role in the heart has not been studied to date. Endogenous methylated arginine residues are also known to inhibit NOS enzymes and can be detected in the human circulation. These were first demonstrated within specialised proteins including myelin basic protein (Baldwin & Carnegie, 1971), heat shock proteins (Wang et al., 1982), and nucleolar proteins (Lischwe et al., 1985). These are

thought to arise from different subclasses of protein-arginine methyl transferase (PRMT) enzymes: one primarily catalysing the formation of L-NMMA and symmetric dimethylarginine (SDMA) and the other L-NMMA and asymmetric dimethylarginine (ADMA; Ghosh et al., 1988; Rajpurohit et al., 1992). These endogenous NOS inhibitors are known to limit NOS enzyme activity by competing with L-arginine. Although the factors affecting regulation of L-NMMA, SDMA, and ADMA are not fully understood they are thought to play some role in the pathogenesis of cardiovascular diseases (Dayoub et al., 2003).

4.7. The role of cofactors

Increasing attention is being directed towards the effects of limited nNOS cofactor availability. This is partly because experimentally, this enables nNOS activity to be arrested potently and acutely. However, intriguing novel evidence also suggests that limited BH_4 availability may be a feature of certain cardiovascular disease states inasmuch as this limits vascular-endothelial NO synthesis in murine streptozotocin-induced diabetes (Alp et al., 2003) and in ApoE knockout mice (Alp et al., 2004). Specifically, reduced availability of BH_4 leads to uncoupled NOS activity, where large amounts of superoxide are generated in place of NO (Cosentino et al., 2001).

Similarly, in the absence of haeme, nNOS uniquely retains the ability to catalyse the NADPH-dependent reduction of cytochrome *c* (Klatt et al., 1996); however, binding of BH_4 is impossible since haeme is necessary for dimerisation of NOS, a requirement for its association with BH_4. The relevance of impaired cofactor availability with respect to cardiovascular disease states has not been specifically established for any of the other cofactors. However, some of the experimental consequences of reduced cofactor binding are known from manipulation of the nNOS structural binding sites, for example, FMN-free nNOS (Adak et al., 1999).

5. Functional correlates of neuronal nitric oxide synthase regulation in the heart

5.1. States that upregulate neuronal nitric oxide synthase

Clinically, the question over whether NO can alter cardiac-autonomic balance in favour of a high vagal tone and reduced β-adrenergic responsiveness is of particular interest. Furthermore, there is a pattern of evidence suggesting that many of the functional effects of NO depend on maximising bioavailability in the tissue of interest. The effect of augmented NO bioavailability in the heart, however, is clearly complex since the effects that have been described using NO donors have been highly variable and dependent on numerous factors including dose, the route and duration of administration, and the level of adrenergic stimulation. Furthermore, NO donors do not accurately replicate the temporal and spatial characteristics of endogenous NO signalling because they bypass the numerous regulatory processes governing the NOS enzyme systems. Another specific caveat associated with the use of NO donors is the inability of their acute effects to be sustained in humans chronically. For example, whilst heart rate variability can be increased in both healthy human subjects (Chowdhary et al., 2000) and heart failure patients (Chowdhary et al., 2002) by the infusion of NO donors acutely, attempts to chronically improve autonomic balance in heart failure patients with transdermal nitroglycerin patches failed (Buch et al., 2004), despite producing significant quantities of the active metabolite in the circulation (Hutt et al., 1994). The reasons for this may be related to the promiscuous nature of NO donors which fail to target the site-specific actions which are carried out by endogenously synthesised NO (Danson, 2004). In addition, chronic administration of nitrates results in augmented tissue oxidative stress and nitrate tolerance which are not necessarily consequences of augmented NOS activity.

According to recent reports, increases in nNOS expression can be transiently induced in response to MI in both parasympathetic neuronal tissue (Takimoto et al., 2002; see Table 2) and ventricular myocytes (Damy et al., 2003). This is associated with increased parasympathetic regulation of heart rate in atria and decreased β-adrenergic signalling in ventricular myocytes, both of which are important positive prognostic indicators against ventricular arrhythmia (Cohn et al., 1984; La Rovere et al., 1998). Short-term ischaemia (<5 min) also activates nNOS activity (Xuan et al., 2000), and this may play an important protective role in ischaemia-reperfusion injury (Jones et al., 2000). Although the precise mechanism through which this operates is not known, it is thought that it involves rapid allosteric modulation of nNOS protein (for review, see Nandagopal et al., 2001). Similar effects on eNOS activity are induced by ischaemia, and therefore a common activating pathway, such as one involving HSP 90, may be important in eliciting this response. In contrast, ischaemia in the rat forebrain (Osuka et al., 2002) or hippocampus (Yan et al., 2004) attenuates nNOS activity by serine phosphorylation.

Significant evidence also exists that chronic exercise is associated with increases in nNOS mRNA and protein in both parasympathetic (Danson & Paterson, 2003; see Fig. 3) and sympathetic neural tissue (Mohan et al., 2000). These effects are associated with vagotonic and sympatholytic effects in isolated preparations of the cardiac atria with intact peripheral nerves. The length of time for which any of these responses can be sustained in living mammals is yet to be demonstrated, as are the physiological and molecular promoters controlling them. Some have speculated that HSP 90 is involved in the nNOS response to MI (Damy et al., 2003) implying that this effect may be an acute phase response in cardiac tissue (see Fig. 4B). The authors of this study also speculated that translocation or

Table 2
Physiological regulation of nNOS activity

Intervention	Tissue	Response	Effects:	Basal	Cholinergic	β-Adrenergic
Exercise training	Parasympathetic ganglia	Increased nNOS protein expression (Danson et al., 2002)		No effect	Increased chronotropic effect (presynaptic)	–
	Stellate ganglion	Increased nNOS protein expression (Mohan et al., 2000)		No effect	–	Small decreased chronotropic effect (presynaptic)
Myocardial infarction	Parasympathetic ganglia	Increased nNOS protein expression (acute response) (Takimoto et al., 2002)		–	Increased chronotropic effect (presynaptic)	–
	LV myocytes	Increased nNOS protein expression Increased caveolin-3/nNOS coprecipitation Increased hsp90/nNOS coprecipitation (Damy et al., 2003)	Inhibition to preserve normal dP/dt, max (in vivo)		–	–
Dilated Cardiomyopathy	LV myocytes	Increased nNOS protein expression Increased caveolin-3/nNOS coprecipitation (Bendall et al., 2004)		No effect (in vitro)	–	Decreased inotropic and elastance effects
	Hypothalamus Dorsal pons Dorsal medulla	Decreased nNOS protein expression (Patel et al., 1996)		–	–	Increased sympathetic outflow
Hypertension	LV myocytes	Increased nNOS, hsp90 protein expression Decreased caveolin-1 and -3 protein expression (Piech et al., 2003)		–	–	–
Chronic angiotensin infusion	LV myocytes	No effect on nNOS protein expression (Moreno et al., 2002)		–	–	–

altered trafficking of nNOS to the sarcolemma was an important step in the modulation of nNOS function in response to MI and have recently demonstrated similar effects in failing human left ventricle (Damy et al., 2004). This may in turn reduce NO modulation of the RyR and calcium spark frequency and augment coupling between NO and the L-type calcium channel by increasing localisation of nNOS to caveolin-3, to which eNOS is normally associated. In these hearts, it is notable, however, that overall NO activity was reduced, owing to a decrease in eNOS expression.

How the effects of exercise training on nNOS-NO signalling compares with that of MI or heart failure therefore is unclear, and both may involve a number of complex molecular mechanisms tailoring nNOS-NO synthesis to modulate function in a specific way. The time course of the pathophysiological effects of nNOS after MI or during heart failure, compared to those of exercise training are also, as yet, unclear, but may differ according to the posttranslational mediators of the response.

5.2. States that down-regulate neuronal nitric oxide synthase

There is less data available relating to pathophysiological stimuli inducing decreases in nNOS expression compared to those documenting increases in nNOS expression in the heart. In neurones, however, activity and nNOS expression are thought to be directly related (Tascedda et al., 1996), and heart failure (although acutely increasing nNOS expression in LV myocytes; Damy et al., 2004) is thought to decrease expression of nNOS in the paraventricular nucleus and brainstem (Patel et al., 1996). This effect is associated with augmented sympathetic outflow to the heart and kidneys in experimental heart failure in the rat (Liu & Zucker, 1999) and can be mimicked by delivery of nNOS antisense into the paraventricular nucleus (Wang et al., 2004) or offset by exercise training (Liu et al., 2000). The changes in nNOS expression in the CNS during heart failure may be related to changes in plasma angiotensin II levels (Liu and Zucker,

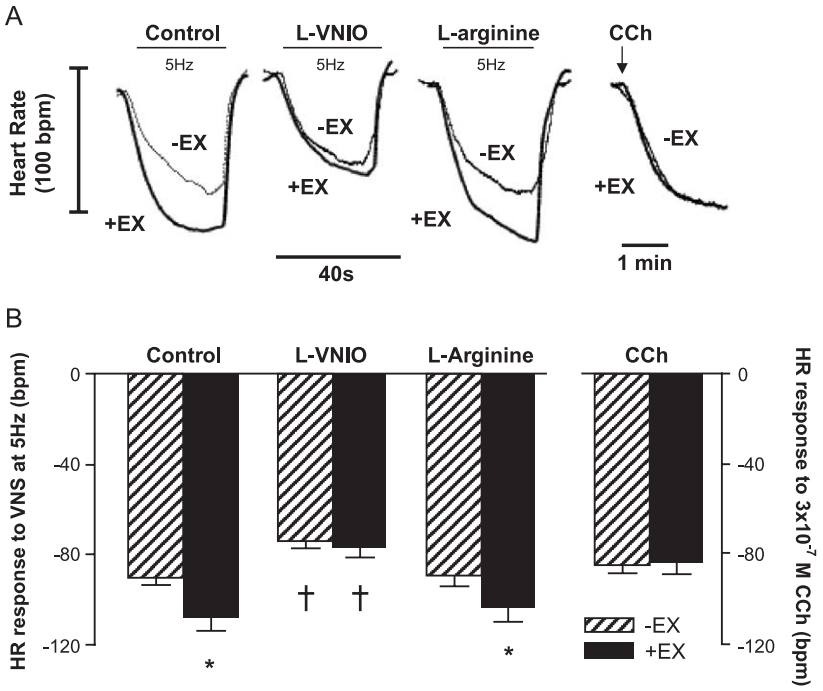

Fig. 3. Adapted from Danson and Paterson (2003). (**A**) Raw data traces show heart rate (HR; bpm = beats min^{-1}) changes with vagal nerve stimulation (VNS) at 5 Hz. HR responses were increased in +EX compared to −EX atria. Responses were normalised in +EX with respect to −EX atria following nNOS inhibition with L-VNIO (100 μmol/L)—an effect which was reversed by excess L-arginine. HR responses to carbamylcholine chloride (CCh, 0.3 μM) were not different in −EX and +EX. (**B**) Graph shows quantitative changes in HR with VNS or CCh. (*P<0.05, unpaired t test; †P<0.01, repeated-measures ANOVA).

1999), infusion of which augments protein expression and nNOS activity in numerous tissues (Moreno et al., 2002), although protein elevations were not seen in the rat LV. The molecular mechanisms through which any of these effects operate, however, are not clear and have not been extensively investigated.

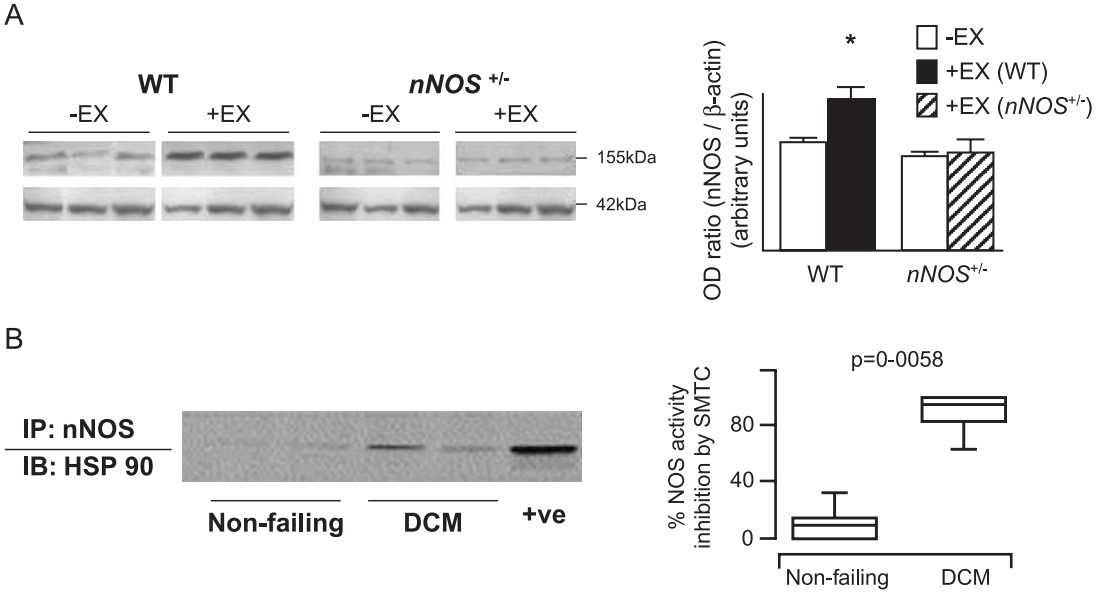

Fig. 4. (**A**) Adapted from Danson et al. (2004). Western blot analysis of homogenised atria showing elevated nNOS protein levels in wild-type (WT) mouse atria following 5 weeks voluntary exercise training (+EX) compared to WT, −EX (*P<0.05, unpaired t test). This effect is absent in atria from $nNOS^{+/-}$ mice. Protein levels are controlled for by β-actin immunoblot. Bar graph illustrates significant changes in nNOS band densities (OD) with respect to β-actin. (**B**) Adapted from Damy et al. (2004). Western blot analysis of homogenised left ventricular (LV) myocytes showing increased immunoprecipitation of nNOS with its allosteric activator heat shock protein 90 (HSP 90) in failing LV myocytes (DCM) compared to nonfailing and a positive nNOS control (+ve). Bar graph illustrates difference in effect of the specific nNOS enzyme inhibitor SMTC on enzyme activity in DCM and nonfailing myocytes.

Also of particular physiological relevance to cardiac pathology is the consequence of increased oxidative stress on nNOS function. It is well known that chronic oxidative stress, including the persistent elevation of superoxide, H_20_2, and other ROS, are features of many cardiovascular disease states including dilated cardiomyopathy and myocardial ischaemia (Lopez Farre & Casado, 2001). Chronic oxidative stress in the rostral ventrolateral medulla (RVLM) of the brainstem results in enhanced sympathetic outflow to the heart. Furthermore, microinjection of SOD into the RVLM can induce a reduction in sympathetic outflow by 70% (Zanzinger & Czachurski, 2000). Given the postulated roles of nNOS, whether similar consequences can be expected from elevated oxidative stress in the myocardium or its neural plexus is an interesting, yet unanswered question.

5.3. Alternate expression, trafficking, and regulation of neuronal nitric oxide synthase

Many of the more recent studies investigating changes in the expression of nNOS in response to physiological stimuli have documented that changes in protein trafficking and regulation were observed along with changes in the absolute protein levels (e.g., Damy et al., 2003). Others have also observed that levels of cardiac nNOS protein do not always correlate with absolute NO bioavailability (Piech et al., 2003) and that fluctuation in protein levels may in fact reflect a mechanism to preserve NO levels in the face of prolonged increased oxidative stress. This concept must be taken into consideration in the interpretation of future data relating to cardiac-nNOS regulation, especially with respect to pathophysiological states where the microenvironment of cardiac tissue undergoes complex and diverse biochemical changes.

6. Dysfunctional neuronal nitric oxide synthase gene regulation

Genomic variation in the nNOS gene and its promoters are liable to predispose certain individuals to different nNOS gene regulatory responses. Although to date this idea has not been specifically established in the heart, it has been shown in other organ systems. In particular, a single-

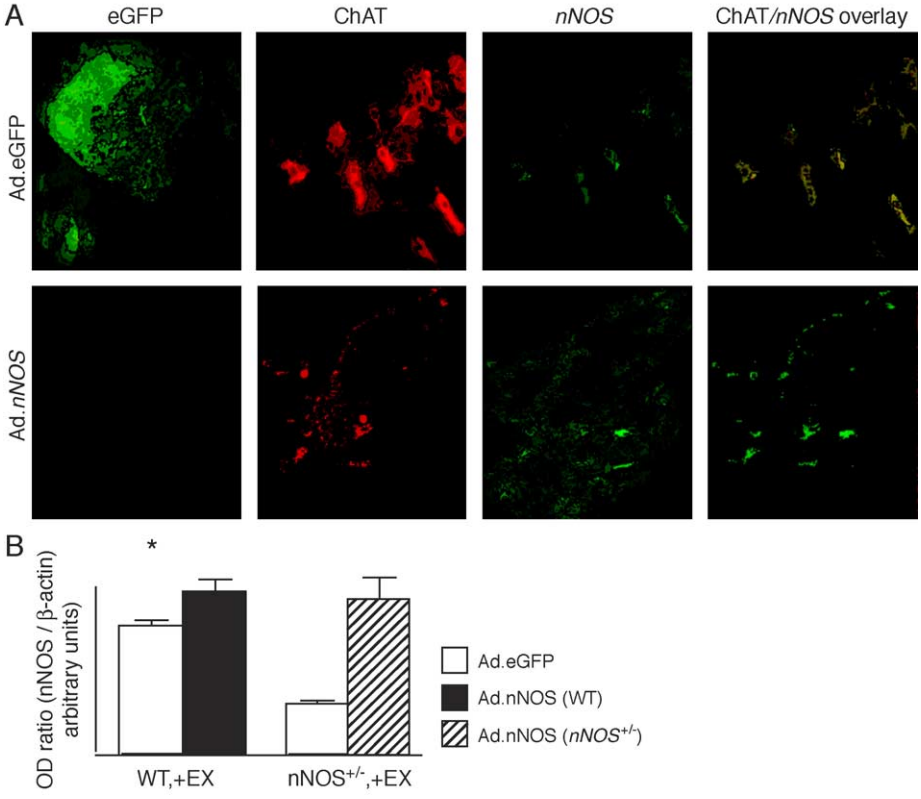

Fig. 5. Adapted from Danson et al. (2004). (**A**) Confocal micrograph illustrates enhanced green fluorescence (far left, green panels) immunoreactivity against choline acetyltransferase (ChAT, middle, red panels) and nNOS (far right, green panels) in atria from $nNOS^{+/-}$, +EX mice pretreated with adenoviruses containing either *eGFP* (top row) or *nNOS* genes (bottom row). The panels show the presence of enhanced green fluorescence located with ChAT-positive cholinergic ganglia in an Ad.*eGFP* specimen with some neurones coexpressing nNOS. In the Ad.*nNOS* specimen, enhanced green fluorescence is not present, and coexpression of nNOS in cholinergic neurones is increased with respect to the Ad.*eGFP* group. (**B**) Western blot analysis showed expression of nNOS in wild-type, +EX; and $nNOS^{+/-}$, +EX atria treated with Ad.*nNOS* or Ad.*eGFP* adenovirus. Ad.*nNOS* significantly increased atrial expression of nNOS protein in both groups (*P<0.001, unpaired t test).

nucleotide gene polymorphism in the nNOS promoter decreases nNOS expression in infantile hypertrophic pyloric stenosis, an effect which may impinge upon the development of the disease (Saur et al., 2004). In addition, other gene polymorphisms have been found which are significantly associated with depression (Yu et al., 2003), Parkinson's disease (Levecque et al., 2003), and acute chest syndrome in sickle cell patients (Sullivan et al., 2001), although whether these correlate with specific patterns of nNOS protein levels is not known. Heterozygous exon 2 nNOS knockout mice also display impaired regulation of nNOS in cardiac parasympathetic ganglia in response to exercise training (Danson et al., 2004, see Fig. 4A). This effect is accompanied by a failure to enhance vagal regulation over heart rate in vivo and in vitro, suggesting that single allele mutations may significantly impinge upon cardiac homeostasis during stress.

7. Alternative mechanisms of neuronal nitric oxide synthase regulation

At present, experimental techniques are advancing in order to develop artificial regulation of nNOS-NO synthesis. Gene transfer of nNOS protein to cardiac parasympathetic ganglia (Mohan et al., 2004; see Fig. 5) and brainstem nuclei (Wang et al., 2003) has been successfully implemented to enhance cholinergic regulation of heart rate and decrease sympathetic outflow, respectively. To date, first generation adenoviruses have been exclusively used as a means of augmenting nNOS expression and activity. These have the disadvantages that they stimulate inflammatory responses, do not target specific cell types, and that expression of transgenes are only maintained for a short period of time. A new generation of adenoviral and lentiviral gene vectors are currently being developed. These have the advantage of carrying larger promoters, allowing exclusive expression in target cell types alone and maintaining longer-term gene expression. Longer-term expression of nNOS and replication of the acute effects already achieved in the modulation of autonomic balance over the heart (Danson et al., 2004) is an important goal in this field of research. Whether the dramatic effects chronic augmentation of vagal-heart rate regulation on mortality in postinfarct rats by electrical nerve stimulation (Li et al., 2004) can be replicated by targeted nNOS gene therapy is a particularly interesting hypothesis to test.

8. Conclusion

There remain numerous unanswered questions relating to the role of nNOS in the control of cardiac function. However, because of what is known about the diversity of regulatory mechanisms impinging on nNOS functionality, describing this role may not be a simple undertaking. What is clear is that nNOS is a dynamic regulatory protein within both cardiomyocytes and the neurones controlling them and that the properties that it possesses make it potentially an important mediator of cardiac homeostasis during stress. As understanding of nNOS in the heart evolves, techniques which enable manipulation of enzyme activity and function may prove useful in therapeutic control of disease processes.

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