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Nitric oxide control of cardiac function: is neuronal nitric oxide synthase a key component?

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Nitric oxide (NO) has been shown to regulate cardiac function, both in physiological conditions and in disease states. However, several aspects of NO signalling in the myocardium remain poorly understood. It is becoming increasingly apparent that the disparate functions ascribed to NO result from its generation by different isoforms of the NO synthase (NOS) enzyme, the varying subcellular localization and regulation of NOS isoforms and their effector proteins. Some apparently contrasting findings may have arisen from the use of non-isoform-specific inhibitors of NOS, and from the assumption that NO donors may be able to mimic the actions of endogenously produced NO. In recent years an at least partial explanation for some of the disagreements, although by no means all, may be found from studies that have focused on the role of the neuronal NOS (nNOS) isoform. These data have shown a key role for nNOS in the control of basal and adrenergically stimulated cardiac contractility and in the autonomic control of heart rate. Whether or not the role of nNOS carries implications for cardiovascular disease remains an intriguing possibility requiring future study.

Keywords: neuronal nitric oxide synthase; cardiac; contraction; autonomic

1. INTRODUCTION

Nitric oxide (NO) has been implicated in the control of cardiac function for well over 10 years. However, despite the extensive literature on this topic, the exact site and nature of action of NO in the heart have remained elusive. Part of the explanation for this may lie in the fact that there are varied and distinct roles for NO in several aspects of myocardial control. These appear to be subserved by specific subcellular localizations and regulation of the different enzymes involved in the production of NO and in their target proteins.

NO is formed through the conversion of L-arginine+O_2 to NO+citrulline, via the action of the enzyme NOS. Thus far, three NOS isoforms have been identified. Two of these, eNOS (or NOS3) and nNOS (or NOS1) are constitutively expressed and are activated by an increase in intracellular calcium (Ca^{2+}). The expression of inducible NOS (iNOS or NOS2), as the name suggests, is induced by inflammatory stimuli and its activity is, in essence, Ca^{2+} independent. The rate of synthesis of NO by eNOS is relatively low compared with that by iNOS and nNOS (in nmol of NO min^{-1}: 16 for eNOS versus 105 for iNOS and 96 for nNOS (Nishida & Ortiz de Montellano 1998)). iNOS can be ubiquitously expressed, and generates high levels of NO, as once iNOS is expressed, it is continuously active (unlike the constitutive NOS isoforms, which are more tightly regulated). Differential expression and regulation of this family of NOS genes and their products is the key for achieving the diversity of action of NO.

In the heart NO has been shown to play a significant role in the control of coronary blood flow and vascular tone, particularly in the mediation of agonist-induced vasodilatation in response to, for example, bradykinin and ACh (e.g. Furchgott & Zawadzki 1980; Moncada *et al.* 1991). eNOS is expressed in cardiac myocytes, and it has historically been this isoform on which attention has focused with regard to the effects of NO on myocardial function. eNOS has been variously suggested to play a role in the control of HR, in contraction, and in protecting against ischaemia/reperfusion injury (e.g. Balligand *et al.* 1993; Han *et al.* 1994; Jones *et al.* 1999; Brunner *et al.* 2003). Many of the studies investigating the role of NO in the heart have used non-isoform-specific inhibitors of NOS, or the exogenous application of NO donors, and confusion has arisen because studies using $eNOS^{-/-}$ mice have not always confirmed the earlier pharmacological findings. More recent data have demonstrated that nNOS is present in the Ca^{2+} store of cardiac myocytes (Xu *et al.* 1999), in peripheral vagal and sympathetic nerves (e.g. Klimaschewski *et al.* 1992; Schwarz *et al.* 1995; Sosunov *et al.* 1996; Choate *et al.* 2001) and in the autonomic control regions of the CNS (e.g. Patel *et al.* 1996), suggesting that this isoform may play a previously unrecognized role in the control of cardiac function and excitability. This review intends to re-assess aspects of the existing literature concerning the role of constitutive NO production in the heart, with a specific focus on the potentially crucial role of the nNOS isoform. The role of iNOS will be mentioned where relevant, but will not be discussed extensively in the review.

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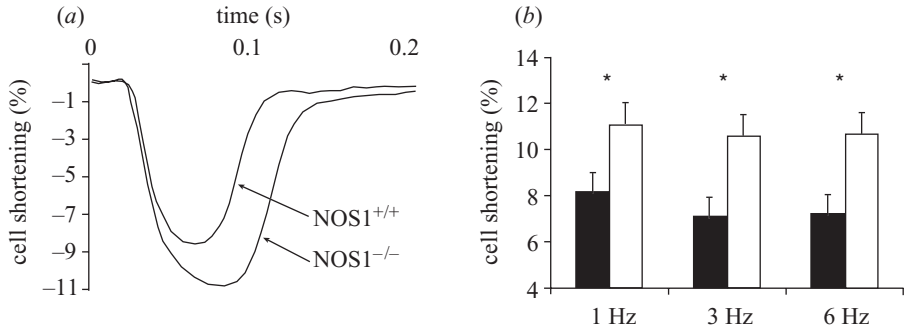

Figure 1. Positive inotropic effect of NOS gene disruption. (*a*) Representative example of cell shortening (expressed as percentage of resting cell length) in field-stimulated LV myocytes from $nNOS^{+/+}$ and $nNOS^{-/-}$ mice. Contraction was significantly greater, and relaxation slowed in the $nNOS^{-/-}$ myocytes. (*b*) This enhanced percentage of cell shortening was seen at 1 Hz, 3 Hz and 6 Hz stimulation frequency. Filled columns, $nNOS^{+/+}$ myocytes; open columns, $nNOS^{-/-}$ myocytes ($^{*}p < 0.05$). (Adapted from Ashley *et al.* 2002.)

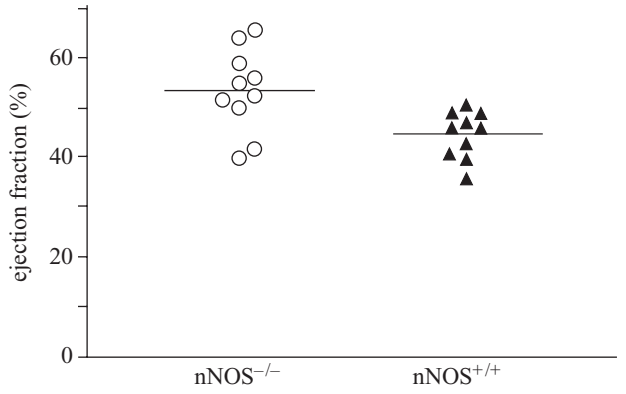

Figure 2. Contractility is enhanced *in vivo* in $nNOS^{-/-}$ mice. Scatter plot to show data for LV ejection fraction in $nNOS^{-/-}$ (open circles) and control (filled triangles) mice. Note that ejection fraction was significantly greater in the $nNOS^{-/-}$ mice. (Adapted from Sears *et al.* (2003*a*).)

2. PROPERTIES OF NEURONAL NITRIC OXIDE SYNTHASE: EXPRESSION, ACTIVITY AND LOCALIZATION

The nNOS gene is extremely complex, with its expression being regulated by a number of alternative first exons, resulting in the production of alternatively spliced nNOS mRNA (Wang *et al.* 1999). A number of splice variants of the nNOS protein have been identified. For example, nNOSμ is a variant 102 nucleotides larger than the nNOS isoform usually found in the CNS (nNOSα), which is expressed in skeletal muscle and possibly in the heart (Silvagno *et al.* 1996; Lin *et al.* 1998*a*). At least six other splice variants are known (Brenman *et al.* 1997), e.g. nNOSβ and nNOSγ, although their physiological function is as yet uncertain. The $nNOS^{-/-}$ mouse was first developed by Huang *et al.* (1993) by ablation of exon 2 of the nNOS gene. The mice are viable and fertile and without evident histological abnormalities in the CNS. However, they do show a pyloric stenosis and hence enlarged stomachs. This may account for the slightly reduced body weight that becomes apparent with age (C. E. Sears and E. A. Ashley, unpublished observation). The $nNOS^{-/-}$ developed by Huang *et al.* still expresses nNOSβ and nNOSγ (which lack the protein-binding domains of nNOSα).

Recently, a mouse lacking exon 6 of the nNOS gene was produced (Gyurko *et al.* 2002), which displayed no alternative splice variants; interestingly these mice were infertile and showed hypogonadism, implicating nNOS in the normal hormonal regulation of reproductive function.

(a) *Localization and regulation of neuronal nitric oxide synthase*

The localization of nNOS within the cell appears to be dependent upon scaffolding adaptor proteins. In skeletal muscle, nNOS forms a complex with dystrophin and α-syntrophin, with binding occurring via the PDZ domain of the α-syntrophin protein and that domain of nNOS (Brenman *et al.* 1995). α-Syntrophin is also found in cardiac muscle (Ahn *et al.* 1996), although its relationship with the cardiac nNOS protein is not known. In neuronal cells the binding of nNOS to NMDA receptors is via a similar PDZ–PDZ interaction (Christopherson *et al.* 1999). In addition, nNOS binds to caveolin-3 in skeletal muscle (Venema *et al.* 1997), as does eNOS, and limited evidence suggests that caveolin may have an inhibitory effect on nNOS (see Michel & Feron 1997), as has been reported for eNOS. There are also suggestions of an interaction of nNOS with caveolin-1 in the canine lower oesophageal sphincter and in vascular smooth muscle (Daniel *et al.* 2001; Cheah *et al.* 2002). Targeting of NOS isoforms to particular subcellular locations is of key importance, exemplified by data showing that the chronotropic response to muscarinic agonists was lost after transfection of an eNOS mutant whose ability to localize to caveolae was disrupted (Feron *et al.* 1998). In addition, the mutant *mdx* mice which lack dystrophin also show very much reduced myocardial nNOS activity (Bia *et al.* 1999). These observations lend further weight to the growing body of evidence that subcellular localization of NOS isoforms subserves their functionality. As already mentioned in § 1, this point may be of importance when considering the results of studies using NOS over-expression or NO donors, where the precise subcellular localization of NO production may be lost.

How nNOS activity is regulated physiologically within ventricular myocytes has not yet been clearly defined. It appears that phosphorylation is less of a factor in the control of nNOS activity than it is for eNOS. eNOS is phosphorylated and activated by the protein kinase Akt

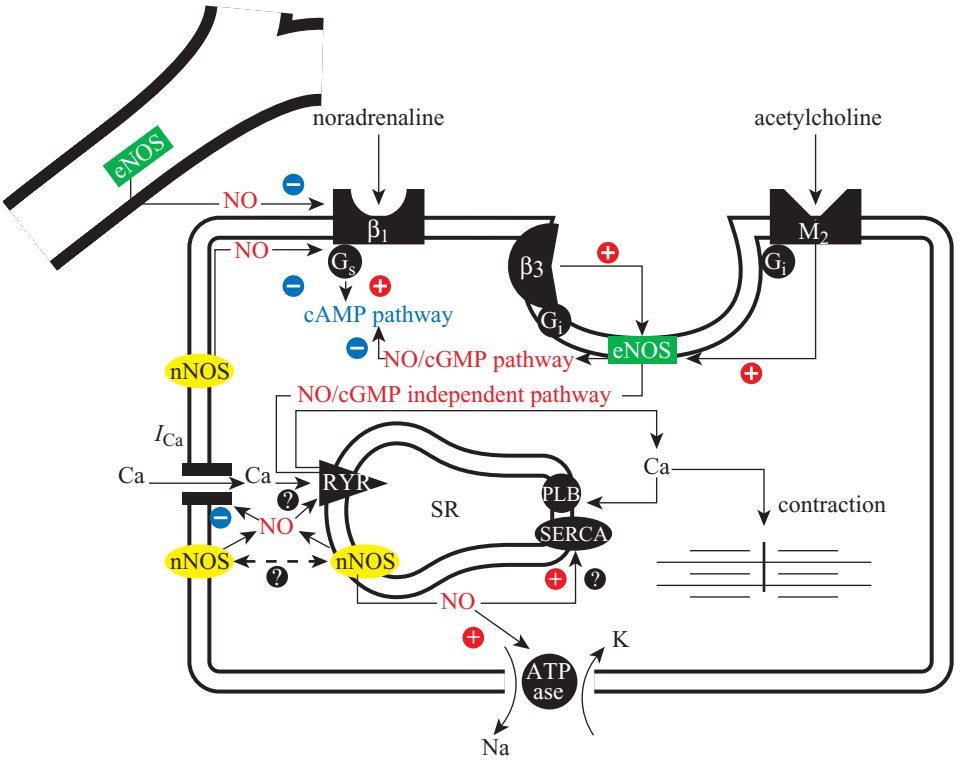

Figure 3. Intracellular NOS pathways involved in the regulation of myocyte contraction and calcium handling. This schematic illustrates the major components of the process of excitation–contraction coupling, whereby Ca^{2+} enters the cell via the calcium current (I_{Ca}), leading to an explosive release of Ca^{2+} from the SR. The resulting rise in intracellular Ca^{2+} switches on the contractile machinery and contraction occurs. This diagram summarizes some of the effects of nNOS and eNOS on myocyte function, both under basal conditions and under adrenergic or cholinergic stimulation. For a detailed discussion see the text. Note that nNOS and eNOS within the myocyte may act in conjunction with NO produced by eNOS from the vasculature. G_i, inhibitory G protein; G_s, stimulatory G protein; PLB, phospholamban; M_2, muscarinic ACh receptor; β_1 and β_3, adrenergic receptors. + (red) denotes a stimulatory action, − (blue) denotes an inhibitory effect. The dashed line between nNOS proteins in the sarcolemma and the SR denotes the potential translocation of the protein under certain conditions (Damy *et al.* 2003).

(Dimmeler *et al.* 1999; Fulton *et al.* 1999). Although nNOS contains an analogous phosphorylation motif, Akt has no effect on NO release by nNOS (Fulton *et al.* 1999). Also, while nNOS can be phosphorylated by protein kinase A (PKA) both *in vitro* and *in vivo* (Bredt *et al.* 1992; Dinerman *et al.* 1994), it is unclear whether this has any significant effect on the activity of the enzyme (Adak *et al.* 2001). In addition, it has been suggested that PKA inhibitors may bind to the endogenous inhibitor protein of nNOS (Jaffrey & Snyder 1996; Yu *et al.* 2002). However, similar to eNOS, heat shock protein 90 (HSP90) stimulates nNOS activity by enhancing the binding of Ca^{2+}/calmodulin to the enzyme (Song *et al.* 2001*a*).

nNOS has been shown to have a very close relationship with Ca^{2+}. For instance, nNOS transcription is regulated by Ca^{2+} (Sasaki *et al.* 2000), and its activity is dependent upon Ca^{2+}. Biosynthesis of NO involves electron transfer from the C-terminal (flavin domain) to the N-terminal (haem domain) end of the enzyme. The binding of Ca^{2+}/calmodulin is thought to trigger this electron transfer (Abu-Soud & Stuehr 1993; Abu-Soud *et al.* 1994) via the displacement of an autoinhibitory insert (Weissman *et al.* 2002). In addition, nNOS co-localizes with Ca^{2+} handling proteins; for instance, in neurons, nNOS is anchored to NMDA receptor-Ca^{2+} channels (reviewed in Baranano & Snyder 2001). Several studies have demonstrated nNOS immunostaining within cardiac myocytes (Brahmajothi & Campbell 1999; Planitzer *et al.* 2002). Specifically, nNOS has been localized to the sarcolemma (Xu *et al.* 2003) and the SR (Xu *et al.* 1999), where it has been shown to be in close proximity to the RyR Ca^{2+} release channel (Barouch *et al.* 2002) and/or the SERCA (Xu *et al.* 1999). A recent study by Damy *et al.* (2003) suggested that in disease states nNOS may 'shuttle' from the SR to the sarcolemma, as evidenced by co-staining of nNOS with the sarcolemmal marker vinculin in a rat model of myocardial infarction. Like eNOS, nNOS is probably inactive at basal levels of intracellular Ca^{2+} in the heart (*ca.* 100 nM) but its activity increases as Ca^{2+} levels rise, with the concentration giving half-maximal effect (EC_{50}) for this reaction being 250–350 nM (Bredt & Snyder 1990; Schmidt *et al.* 1991).

Cardiac mitochondria also contain nNOS (Kanai *et al.* 2001; Elfering *et al.* 2002). This is a potentially interesting finding, as NO has been shown to inhibit cellular respiration (Borutaite & Brown 1996; Brown *et al.* 1997). However, as the activity of the mitochondrial isoform seems to be extremely low in the heart, and NOS inhibitors had no effect on rate of respiration in isolated porcine mitochondria (French *et al.* 2001), the physiological implications of this localization remain unclear.

The tissue-specific expression of different regulatory factors and indeed the expression of the different nNOS splice variants will clearly affect the role of nNOS-derived

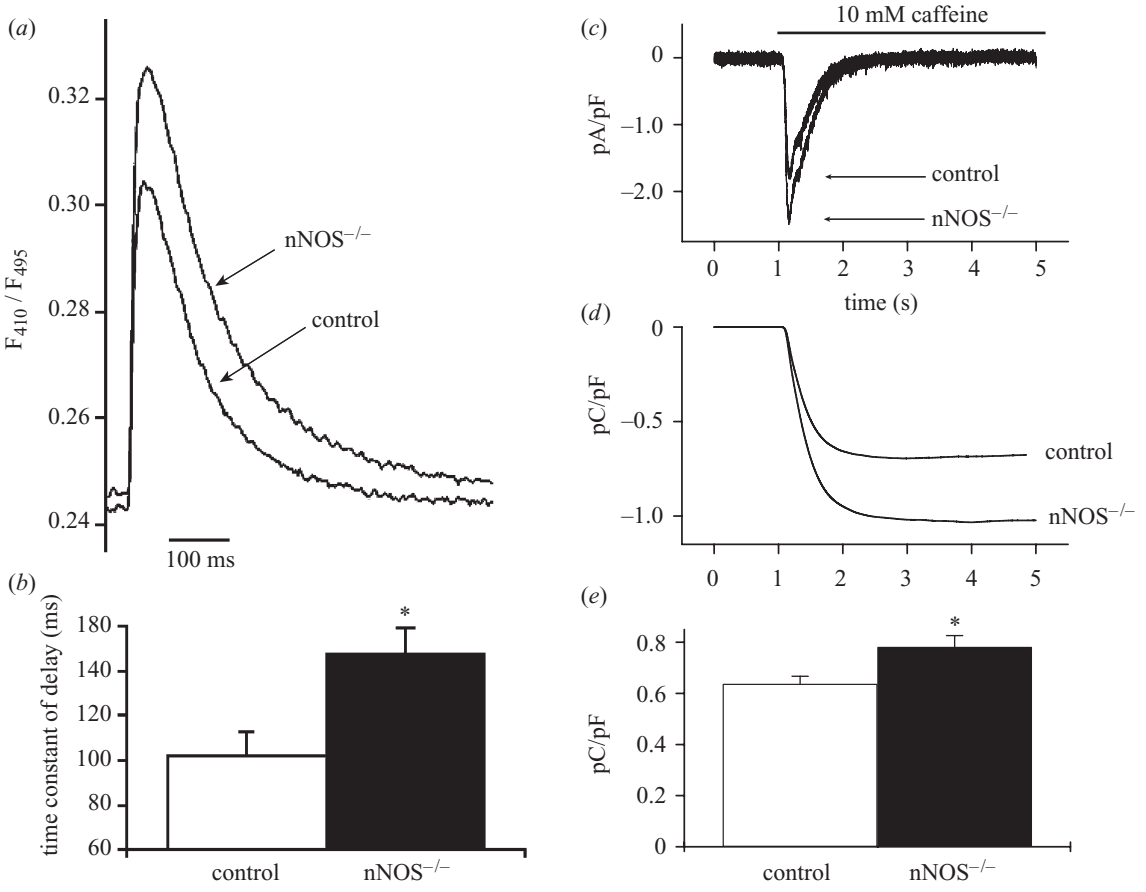

Figure 4. Calcium transients and SR load are enhanced in myocytes from nNOS$^{-/-}$ mice. (*a*) Average raw data trace showing the indo-1 fluorescence ratio (410/495 nm) in control and nNOS$^{-/-}$ myocytes. Transients recorded from nNOS$^{-/-}$ myocytes had greater peak fluorescence. (*b*) The time-course of decay of the Ca^{2+} transient was significantly slower in the nNOS$^{-/-}$ than in control myocytes ($^{*}p < 0.05$). (*c*) Example records of currents (in pA/pF) elicited in nNOS$^{-/-}$ and control myocytes by a 10 s exposure to 10 mmol l^{-1} caffeine. These currents are carried predominantly via the sodium–calcium exchanger in Ca^{2+}-extrusion mode. (*d*) Integral of the currents in (*a*) in pC/pF. (*e*) Average results of caffeine-induced current integrals, indicating a larger SR load in nNOS$^{-/-}$ myocytes ($^{*}p < 0.05$). (Adapted from Sears *et al.* (2003*a*).)

NO in the various tissues and cell types in which it is expressed, as may the availability of cofactors and substrates. It is entirely conceivable that this labile and diffusible gas may have disparate roles within even one cell. In the myocardium this may be particularly pertinent because of the presence of myoglobin, which as a scavenger of NO could limit the scope of action of NO derived from a particular NOS isoform to a subscribed region within the cell (Flogel *et al.* 2001).

3. POTENTIAL POINTS OF CONTROVERSY

It will be apparent to anyone acquainted with the literature concerning the effects of NO on the heart that it abounds with conflicting data, and also that the reasons behind some of the controversies in the field remain obscure. However, before we move on to the body of the review considering the literature on the NO control of cardiac function there are a few points which may be useful to bear in mind, most of which will be expanded upon, with examples given throughout the review.

(i) One important factor is the concentration of NO, or of its downstream pathway components (e.g. cGMP, PKG) that are present in the experimental system. Many studies have used NO donors (or NO intracellular signalling molecules), which may result in intracellular concentrations of NO or cGMP that are very different to those seen under normal physiological conditions. In addition, some studies using overexpression of NOS have produced very high NO levels (e.g. Brunner *et al.* 2001). Concentration-dependent effects of NO have been demonstrated in a number of studies (e.g. Mohan *et al.* 1995; Wyeth *et al.* 1996; Vila-Petroff *et al.* 1999). Thus, drawing a distinction between what NO *can* do and what it does under normal physiological conditions may be useful. NO donor studies may be useful when considering the effects of NO under circumstances when the NO system is driven, for example, in certain disease states or in exercise.

(ii) NO donors also vary in the rate of NO release, the extent of 'side reactions' such as nitrosation of proteins, and the production of by-products, and thus the choice of NO donor made by an investigator may have a bearing on the results obtained (for further discussion see Feelisch (1998)).

(iii) It has been suggested that the specific subcellular location of the NOS isoforms and their effector

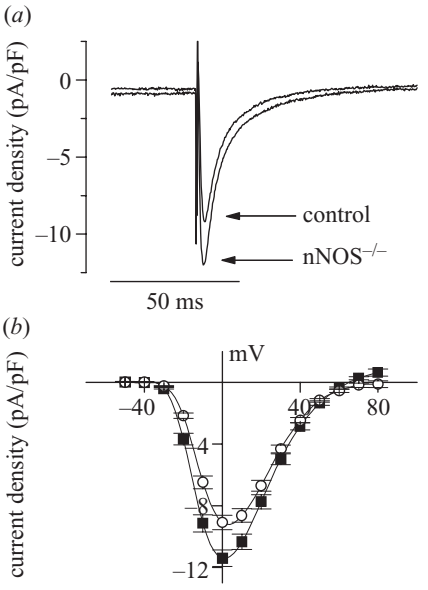

Figure 5. Calcium currents from nNOS$^{-/-}$ myocytes are larger and slower to decay. (*a*) Example records of I_{Ca} (in pA/pF) elicited by a 200 ms depolarizing step from −40 mV to 0 mV in control and nNOS$^{-/-}$ myocytes. (*b*) Note that the current–voltage relationship shows I_{Ca} density is greater in nNOS$^{-/-}$ myocytes (filled squares) than in controls (open circles) over the physiological voltage range ($p < 0.05$). (Adapted from Sears *et al.* (2003*a*).)

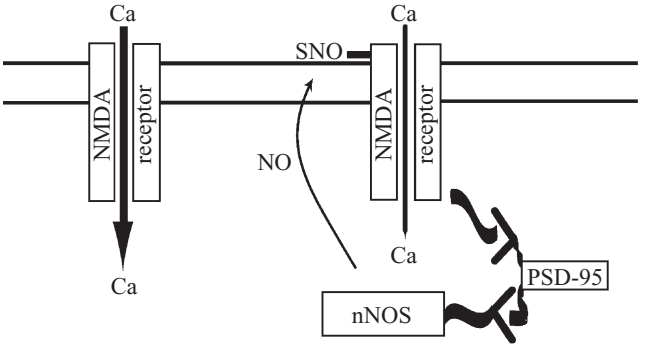

Figure 6. Negative feedback loop for nNOS control of Ca^{2+} entry through the NMDA receptor. Schematic diagram to illustrate Ca^{2+} entry through the NMDA receptor, coupled to the nNOS protein via PSD-95. nNOS is activated as Ca^{2+} rises in the cell, and then the nNOS-derived NO in turn acts back on the NMDA receptor to inhibit further entry, via an S-nitrosylation reaction. (Adapted with permission from Baranano & Snyder (2001); copyright © 2001 National Academy of Sciences, USA.)

proteins may be of prime importance (e.g. Barouch *et al.* 2002). These site-specific intracellular effects of NO may well be lost when NO donors or NOS overexpression are used, and this may affect the findings.

(iv) More than one NOS isoform is constitutively present within the myocardium. Both eNOS and nNOS have been shown to be present, and therefore the use of non-isoform-specific NOS inhibitors will not be able to distinguish between the effects mediated by these two isoforms.

(v) Recent years have brought about the advent of gene knockout technology. While selective gene disruption has proved very useful in teasing out the effects of single NOS isoforms on cardiac physiology, these studies need to be approached with caution, as potential upregulation of compensatory pathways may confound the findings. Complementing data from knockout mice with experiments using isoform specific inhibitors or antisense of the NOS isoform in question, as well as carrying out conditional knockouts of the genes of interest, or gene 'knock-in' experiments, may be useful in clarifying this issue.

(vi) Experimental conditions also need to be taken into account. Conditions such as temperature are key, particularly when the studies involve investigations into enzymes and Ca^{2+} handling proteins, which are exquisitely dependent upon temperature (e.g. Allen 1996). Also, the frequency of stimulation may affect results, for example Kaye *et al.* (1996) showed that NO production increases with the stimulation frequency in ventricular myocytes. To this end, studies in the mouse should bear in mind that the mouse heart normally beats at 500–600 b.p.m. The type of cell and species may also affect the results obtained, for example, atrial versus ventricular, and mammalian versus amphibian.

(vii) Specifically with regard to mouse studies, it is potentially of interest that the strain of mouse used may affect experimental results. This is important for studies in NOS knockout mice, where it is best to use siblings as the control animals, as they construe the closest genetic and environmental match to the knockout mice.

(viii) When interpreting the results of *in vivo* studies, particularly those using non-isoform-specific NOS inhibitors, the effects of these agents on coronary flow should be noted. This may be of importance when NOS inhibition is administered via the coronary circulation, where changes in coronary flow may affect the concentration of the agent 'seen' by the myocardium. Also, for *in vivo* studies the method of measuring the index of contractility, the anaesthetic technique, and the haemodynamic status and HR of the animal should be taken into consideration when comparing findings from different groups (reviewed by Kass *et al.* 1998). For example, some indices of LV function, such as ejection fraction, are load dependent and will therefore be affected by factors such as arterial BP, HR and end-diastolic volume. The significant change in BP caused by chronic use of non-isoform specific NOS inhibitors or by eNOS gene disruption, and the subsequent development of LV hypertrophy (Huang *et al.* 1995; Godecke *et al.* 1998), may confound the changes attributable to the decrease in eNOS activity *per se*, and may therefore affect the interpretation of these studies.

4. THE ROLE OF NITRIC OXIDE IN THE BASAL CONTROL OF MYOCARDIAL FUNCTION

Although the paracrine effects of endothelial or endocardial eNOS-derived NO are well established (reviewed

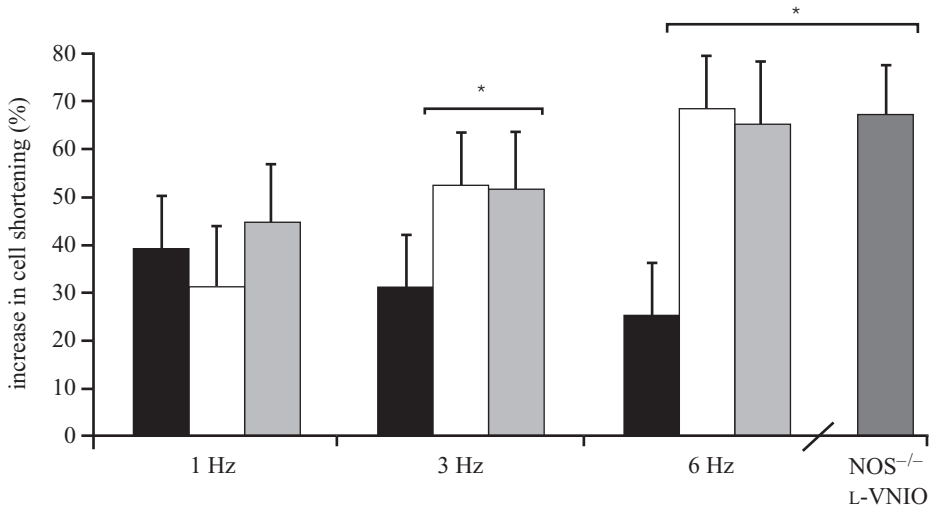

Figure 7. Increase in cell shortening with low-dose isoprenaline is enhanced in $nNOS^{-/-}$ mice. Percentage increase in cell shortening in 2 nM isoprenaline is greater in $nNOS^{-/-}$ myocytes and in $nNOS^{+/+}$ myocytes treated with the nNOS-specific inhibitor, L-VNIO (500 μM), field-stimulated at 3 Hz and 6 Hz. Note that incubation of $nNOS^{-/-}$ myocytes with L-VNIO did not lead to any additional effect. (Adapted from Ashley *et al.* 2002). Black bars, $NOS1^{+/+}$; white bars, $NOS1^{-/-}$; grey bars, $NOS1^{+/+}$ L-VNIO. *, $p < 0.05$.

in Shah 1996), the autocrine effects of NO on basal cardiac function have remained controversial. In this review 'basal' is taken as meaning in the absence of stimulation by agonists or of disease.

(a) *Effects of nitric oxide on basal cardiac contraction*

Although the effect of NO on cardiac contraction has been extensively studied in recent years, a consensus remains elusive. Some studies argue against an effect of NOS inhibition on basal cardiac contraction (Klabunde *et al.* 1992; Balligand *et al.* 1993; Brady *et al.* 1993; Keaney *et al.* 1996; Harding *et al.* 1998). Similarly, there was no effect of NO donors or of stimulation of NOS activity with L-arginine on the baseline contractile function of cultured chick ventricular myocytes (Ungureanu-Longrois *et al.* 1997). These *in vitro* results have also been replicated *in vivo*. For example, in the anaesthetized dog, coronary infusion of non-isoform-specific NOS inhibitors, L-NAME or L-NMMA had no effect on LV inotropy (Crystal & Gurevicius 1996).

Other investigators would argue for an effect of NO on the basal inotropic state of the myocardium. However, it is often the case that these data have been obtained by application of exogenous NO, stimulation of NO release from the coronary endothelium or by manipulation of the intracellular NO–cGMP pathway. Shah *et al.* (1994) demonstrated a negative inotropic effect of 8-br-cGMP (a membrane-permeable analogue of cGMP), via a PKG-dependent reduction in the myofilament responsiveness to Ca^{2+}. A similar negative inotropic action was elicited in field-stimulated guinea-pig ventricular myocytes by the addition of SNP (Brady *et al.* 1993). However, various studies have shown the effect of NO donors on contraction to be concentration dependent, with low doses having a small positive inotropic effect and high doses a negative effect (Mohan *et al.* 1995; Wyeth *et al.* 1996; Vila-Petroff *et al.* 1999). Analogous to addition of high concentrations of NO donors, up to 80-fold cardiac-specific overexpression of eNOS caused some depression of LV developed pressure (Brunner *et al.* 2001).

These data obtained with NO donors and overexpression of NOS, which contradict many of the studies using NOS inhibitors, may therefore be a prime example of a situation where the concentration and mode of delivery of NO have a significant bearing on the experimental results obtained.

There are, however, reports showing an effect of inhibition of endogenous production of NO on cardiac contractility. For instance, L-NMMA was reported to have a positive inotropic effect in isolated hamster papillary muscle (Finkel *et al.* 1995), and in rat ventricular myocytes at frequencies of stimulation of 3 Hz (Kaye *et al.* 1996). Conversely, others have reported a moderate reduction in contractility with coronary infusion of NOS inhibitors *in vivo* or in isolated hearts (Amrani *et al.* 1992; Kojda *et al.* 1997; Cotton *et al.* 2001). However, in the Amrani study, NOS inhibition reduced coronary flow in the isolated working heart preparation, which may have affected the results. It is also worth noting that the positive inotropic effect of NO donors is significantly enhanced by NOS inhibition (Muller-Strahl *et al.* 2000), which may suggest that the positive inotropic action of endogenous NO is near maximal at normal physiological concentrations in hearts with an intact endothelium.

It has often been assumed that any effect of NO on contractility is a result of eNOS-derived NO. As technology has advanced, investigators have been able to take advantage of the development of isoform-specific knockout mice, which circumvents some of the problems of using non-isoform-specific inhibitors of NOS (although one must retain caution concerning the potential upregulation of compensatory mechanisms that may develop throughout ontogeny when a gene is disrupted). While there is strong evidence that stimulation of NO production from the endothelium can modulate LV function, and in particular diastolic function (for review see Shah & MacCarthy (2000) and further discussion later in this review),

it has never been demonstrated that endogenous eNOS-derived NO from within the myocyte has any effect on basal contractility or relaxation. For example, in the study by Gyurko *et al.* (2000) *in vivo* and *in vitro* measures of contractility showed no difference in $eNOS^{-/-}$ mice compared with 'control' animals (in their case a combination of WT littermates of the $eNOS^{-/-}$ mice, 129/SvJae and C57BL6 mice) a result also found by Han *et al.* (1998*a*), Vandecasteele *et al.* (1999), Godecke *et al.* (2001) and Barouch *et al.* (2002). Further, echocardiography of conscious mice also showed no difference in basal function between $eNOS^{-/-}$ and WT animals (Yang *et al.* 1999). Recent evidence would also suggest a limited role for iNOS on the control of basal contractility, as cardiac-specific iNOS over-expression does not result in significant depression of basal cardiac function (Heger *et al.* 2002; Mungrue *et al.* 2002), although this finding may be a 'false negative' and result from NOS substrate depletion. Consistent with this hypothesis, the administration of L-arginine led to a fall in LV pressure in the iNOS transgenic hearts (Heger *et al.* 2002). In addition, it has recently been shown that myoglobin functions as an important buffer of iNOS-derived NO, as iNOS over-expression results in much greater depression of cardiac function in myoglobin knockout mice (Godecke *et al.* 2003; Wunderlich *et al.* 2003).

(b) *Does the neuronal nitric oxide synthase isoform play a role in the control of basal contraction by nitric oxide?*

A potential explanation for the divergent results obtained with non-isoform-specific NOS inhibitors versus $eNOS^{-/-}$ mice could lie in the fact that nNOS is also present in the myocardium (Xu *et al.* 1999). For example, studies from our group have shown that inhibition of nNOS activity, via gene disruption or acute pharmacological blockade, enhanced basal contractility (Ashley *et al.* 2002; Sears *et al.* 2003*a*; figure 1). Importantly, this effect was demonstrated at physiological temperatures and over a range of frequencies, including those relevant to murine myocardium. This is a key consideration, given the increase in NOS activity with increases in stimulation frequency (Kaye *et al.* 1996), and the temperature dependence of many of the processes involved in excitation–contraction coupling (Allen 1996). An enhanced LV systolic function was also seen *in vivo*, where LV ejection fraction was higher in $nNOS^{-/-}$ compared with WT nNOS ($nNOS^{+/+}$) littermates (Sears *et al.* 2003*a*; figure 2), in the absence of potentially confounding differences in HR or BP between strains. These data are in at least partial agreement with those of Barouch *et al.* (2002) who showed that $nNOS^{-/-}$ mice had a higher LV dP/dt_{max} corrected for instantaneous pressure than C57BL/6 mice, although an alternative measure of LV contractility (coupling of LV elastance to arterial elastance) showed no difference between the two groups. However, these investigators did not show any difference in the basal contraction of LV myocytes between $nNOS^{-/-}$ and C57BL/6 mice (Barouch *et al.* 2002; Khan *et al.* 2003).

(c) *Is there an effect of neuronal nitric oxide synthase on myocyte calcium handling?*

The mechanisms underlying the increase in contractility in $nNOS^{-/-}$ mice were investigated in detail in isolated LV myocytes (Sears *et al.* 2003*a*), where we showed that the positive inotropic effect of nNOS inhibition/gene disruption was related to the effects of nNOS on Ca^{2+} handling (see figure 3 for a schematic illustrating some of the intracellular pathways involved). Both I_{Ca} and SR Ca^{2+} load were enhanced in the presence of nNOS gene disruption or pharmacological inhibition (figure 4). The increased entry of Ca^{2+} via I_{Ca} in $nNOS^{-/-}$ myocytes (figure 5) provides an increased stimulus for Ca^{2+}-induced–Ca^{2+}-release and may contribute to the higher SR Ca^{2+} load, resulting in greater Ca^{2+} transient and contraction (figure 3). Further to this, it has been suggested that nNOS-derived NO increases the activity of Na^+–K^+–ATPase pump (Zhou *et al.* 2002; Xu *et al.* 2003), which may indirectly affect Ca^{2+} fluxes through an action on intracellular Na^+ levels, and a resultant change in activity of the Na^+–Ca^{2+} exchanger. Other investigators, however, have not observed such changes in Ca^{2+} handling in the $nNOS^{-/-}$ myocardium. Khan *et al.* (2003) saw no difference in Ca^{2+} transient amplitude or SR Ca^{2+} load in $nNOS^{-/-}$ myocytes compared with C57BL/6; and, in experiments carried out at room temperature, Barouch *et al.* (2002) showed no difference in cell shortening or Ca^{2+} transients in $nNOS^{-/-}$ myocytes. However, there was a tendency for I_{Ca} (recorded at 37 °C) to be greater in $nNOS^{-/-}$ myocytes in Barouch *et al.* (2002) although this was not statistically significant. The reasons behind these discrepancies are unclear, but may relate to the use of C57BL/6 mice as controls in Barouch *et al.* and Khan *et al.* versus $nNOS^{+/+}$ littermates in our experiments.

Effects of NO on I_{Ca} have been studied extensively, as I_{Ca} is the primary point of entry of Ca^{2+} into the myocyte, and the trigger for SR Ca^{2+} release. However, results obtained with non-isoform-specific NOS inhibitors or with addition of exogenous NO on basal I_{Ca} have been variable and have not been supported by studies using $eNOS^{-/-}$ mice. For example, some studies have found no effect of the downstream NO signalling molecule cGMP on basal I_{Ca} over a range of concentrations (Hartzell & Fischmeister 1986; Mery *et al.* 1991; Kumar *et al.* 1997), while in human atrial myocytes the NO and superoxide donor SIN-1 had a biphasic effect on I_{Ca} (Kirstein *et al.* 1995). Others have shown that high concentrations of non-isoform-specific NOS or guanylate cyclase inhibitors have a stimulatory effect on I_{Ca} (Gallo *et al.* 1998, 2001). Tellingly, no difference in basal I_{Ca} has been observed in the $eNOS^{-/-}$ mice compared with WT (Vandecasteele *et al.* 1999; Godecke *et al.* 2001), although methodological concerns remain that have not been investigated in a systematic fashion (e.g. temperature at which experiments were conducted, stimulation frequency and the potential influence of LV hypertrophy in $eNOS^{-/-}$ mice; for a detailed discussion see Balligand (1999)). Nevertheless, our data showing enhanced I_{Ca} in the presence of nNOS gene disruption or acute pharmacological inhibition may stimulate a re-assessment of some of the conflicting literature in this field.

nNOS-derived NO may inhibit I_{Ca} via the NO–cGMP pathway, as inhibition of guanylate cyclase with 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one has been shown to increase peak I_{Ca} (Gallo *et al.* 2001), and PKG phosphorylation has an inhibitory effect on basal I_{Ca} (Sumii & Sperelakis 1995). However, it is also possible that the

mechanism of action of nNOS-derived NO on I_{Ca} might involve a change in the redox state of the channel. Hu *et al.* (1997) showed that *S*-nitrosothiols decrease I_{Ca} independent of cGMP in expressed Ca^{2+} channels although Campbell *et al.* (1996) showed stimulation of I_{Ca} by nitrosothiol agents in isolated myocytes.

Taken together, our data suggest that nNOS-derived NO may play a negative feedback role in regulating Ca^{2+} handling, as increases in intracellular Ca^{2+} stimulate nNOS synthesis of NO, which could then in turn act to inhibit Ca^{2+} fluxes. This feedback regulation of Ca^{2+} entry by nNOS-derived NO is not without precedent. Indeed, a similar mechanism has been proposed for the NO regulation of capacitative Ca^{2+} entry in the endoplasmic reticulum of the vascular endothelium (Dedkova & Blatter 2002). In addition, in neurons, where nNOS is co-assembled with the NMDA receptor, Ca^{2+} influx through this channel activates nNOS (Garthwaite *et al.* 1988), and nNOS-derived NO in turn inhibits Ca^{2+} entry through the NMDA receptor, in a negative feedback loop (figure 6; Kim *et al.* 1999; Baranano & Snyder 2001). It is interesting to speculate that nNOS-derived NO could be involved in the beat-to-beat regulation of myocardial Ca^{2+} fluxes and contraction, especially as the myocardial levels of NO appear to increase and decrease with each cardiac cycle (Pinsky *et al.* 1997).

(d) *Does neuronal nitric oxide synthase regulate the activity of the ryanodine receptor Ca^{2+} release channel?*

nNOS has also been implicated in the control of other proteins involved in Ca^{2+} handling, for example the Ca^{2+} release channel in the cardiac SR, or the RyR. NO has been shown to both enhance (Stoyanovsky *et al.* 1997; Xu *et al.* 1998) and inhibit (Zahradnikova *et al.* 1997) Ca^{2+} release by the RyR, and co-immunoprecipitation experiments indicated that nNOS may form a complex with the RyR (Barouch *et al.* 2002). As many of these studies have been carried out using NO donors on RyR expressed in lipid bilayers, the physiological significance of these findings remains uncertain. Indeed, in isolated rat ventricular myocytes, there was no effect of NO on basal spark frequency, which is thought to be reflective of activity of the RyR (Ziolo *et al.* 2001). Additionally, our data show no difference in the time to peak of the Ca^{2+} transient in $nNOS^{-/-}$ and WT myocytes, suggestive of a lack of effect of nNOS-derived NO on the Ca^{2+} release channel (Sears *et al.* 2003*a*). It should also be considered that an isolated effect of NO on the open probability of the RyR may have limited functional significance in regard to the long-term control of contraction, as such changes are thought to result in only transient effects on contraction (Eisner & Trafford 2000).

(e) *Role of myocardial endothelial nitric oxide synthase in the stretch-mediated increase in contraction*

Myocardial eNOS may be more important in the control of cardiac function when the enzyme is activated by specific stimuli; for example, eNOS appears to play a key role in the cardiac response to stretch. Stretching myocytes causes an increase in the Ca^{2+} transient through NO-mediated stimulation of Ca^{2+} release by the RyR (Petroff *et al.* 2001). This effect appears to be catalysed by phosphorylation of eNOS via the phosphatidylinositol-3-OH and Akt kinases (Petroff *et al.* 2001; figure 3), perhaps in conjunction with an increase in Ca^{2+} entry via the Na^{+}–Ca^{2+} exchanger. This mechanism may underlie the slow increase in cardiac contraction in response to prolonged stretch (the Anrep effect).

(f) *Regional differences in nitric oxide synthase isoform expression*

Bett *et al.* (2002) showed that NO scavenging or soluble guanylate cyclase inhibition had variable effects on I_{Ca} in ferret right ventricular myocytes, either producing no effect or resulting in stimulation of the current. This could be related to the observed regional differences in the expression of nNOS (Brahmajothi & Campbell 1999), as basal expression of nNOS in ferret ventricular tissue was higher in the endocardium than in the epicardium, whereas eNOS expression was higher in the LV epicardium (Brahmajothi & Campbell 1999). The physiological implications of these proposed regional differences have, however, not been studied. It is possible that differences in regional expression of NOS isoforms may explain some controversies in the literature. However, given that the standard methodology for cardiac myocyte isolations involves the entire LV, it seems unlikely that a systematic bias in the yield of epicardial versus subendocardial myocytes between laboratories may account for their differing results. However, there do seem to be differences in expression of NOS isoforms between different tissues, for example Miethke *et al.* (2003) noted more nNOS RNA in atrial than in ventricular tissue.

Thus, the different functions of the NOS isoforms on basal function may be influenced not only by their different localizations but also in their response to different stimuli. Phosphorylation of eNOS in LV myocytes may be responsible for sustained NO release in response to mechanical stimuli whereas nNOS activity may be regulated by fluctuations in intracellular Ca^{2+} on a beat-by-beat basis. It is conceivable that nNOS may have a role to play in the beat-to-beat regulation of basal cardiac function, perhaps as a negative feedback regulator of Ca^{2+} fluxes, whereas eNOS may be more important in situation of stress for the myocyte, for example under increased preload or other forms of mechanical stretch.

(g) *Effects of nitric oxide on basal relaxation*

It has been well established for some years now that NO has a lusitropic effect on the LV myocardium, in that it facilitates LV relaxation and diastolic filling (reviewed in Shah & MacCarthy 2000). In isolated working guinea-pig hearts, coronary infusion of L-NMMA or haemoglobin attenuated the rise in cardiac output caused by increases in preload, but had no effect on baseline cardiac output (Prendergast *et al.* 1997), leading to the conclusion that NO augments the Frank–Starling response by increasing LV compliance. Similarly, the NO donor SNP facilitates basal LV relaxation in isolated guinea-pig hearts (Grocott-Mason *et al.* 1994) and in humans (Paulus *et al.* 1994).

The facilitatory effect of NO on LV relaxation, however, was not confirmed by studies in the $eNOS^{-/-}$ mouse, which shows normal or enhanced LV diastolic function in basal conditions or during β-adrenergic stimulation,

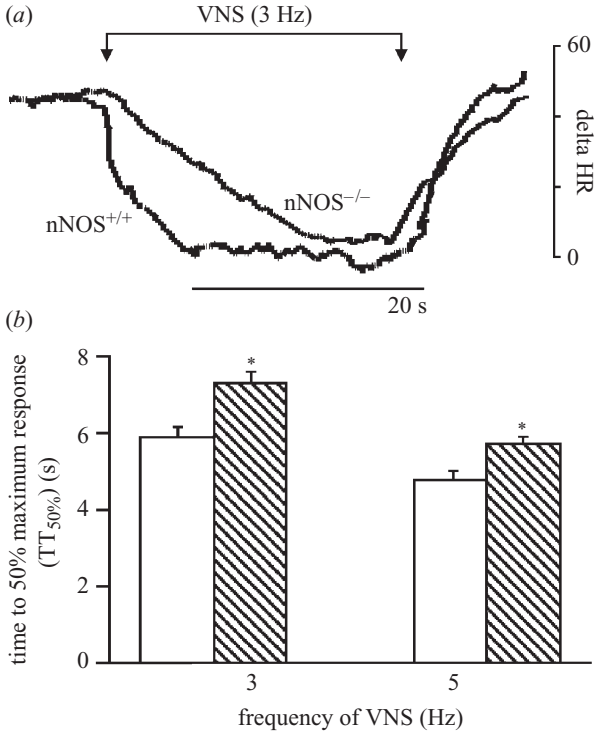

Figure 8. Heart rate response to vagal stimulation is slowed in the nNOS$^{-/-}$ atria. (*a*) Raw data trace showing the fall in HR with 3 Hz vagal nerve stimulation. (*b*) Histogram showing the time-course of the decay (TT50%) of the vagal HR response. Note that the decline in HR is slowed in atria with vagal nerve stimulation (VNS) from nNOS$^{-/-}$ (hatched bars, $n = 56$), compared with their WT littermate controls (nNOS$^{+/+}$) (open bars, $n = 39$) at 3 Hz and 5 Hz stimulation frequency. (Reproduced with permission from Choate *et al.* (2001).)

respectively (Gyurko *et al.* 2000). The authors attributed these findings to a compensatory rise in the expression of atrial natriuretic peptide. An alternative answer may be found in the role of nNOS. We demonstrated a delayed relaxation in the presence of acute pharmacological inhibition or gene disruption of nNOS in field-stimulated LV myocytes (Ashley *et al.* 2002). Similarly, LV relaxation was somewhat impaired *in vivo* in the nNOS$^{-/-}$ mouse (Sears *et al.* 2003*a*). Barouch *et al.* (2002) and Khan *et al.* (2003) did not report an impairment of LV relaxation in basal conditions. However, interestingly, Khan *et al.* (2003) did see an attenuated frequency-related lusitropic response in nNOS$^{-/-}$ mice both in isolated myocytes and *in vivo*, consistent with a role for nNOS-derived NO in the regulation of myocardial relaxation under these conditions.

The effect of NO on LV relaxation may be a result of a cGMP-mediated reduction in myofilament Ca^{2+} sensitivity, possibly following PKG-mediated phosphorylation of troponin I (Kaye *et al.* 1999; Layland *et al.* 2002). Preliminary data from our laboratory suggest, however, that myofilament sensitivity is not altered in nNOS$^{-/-}$ myocytes, as the relationship between peak cell shortening and Ca^{2+} influx in the presence of SR inhibition did not differ between nNOS$^{-/-}$ and WT myocytes (Sears *et al.* 2003*a*). Furthermore, the decay of the $[Ca^{2+}]_i$ transient was slower in nNOS$^{-/-}$ mice, suggesting that nNOS-derived NO may stimulate SERCA activity, as recently indicated by Zhou *et al.* (2002). This hypothesis, however, requires further investigation.

(h) *Effects of nitric oxide on basal heart rate*

Inhibition of NOS with non-isoform-specific inhibitors has been shown to have either no effect on baseline HR (Elvan *et al.* 1997; Conlon *et al.* 1998), or to have a modest bradycardic effect (Pabla & Curtis 1995; Liu *et al.* 1996; Kojda *et al.* 1997; Musialek *et al.* 1999). NO donors (in the nanomolar to micromolar range), however, elicit a 'direct' positive chronotropic effect *in vitro*. We showed an increase in the spontaneous beating rate of isolated guinea-pig atria with several NO donors in the nanomolar to low micromolar concentration range (Musialek *et al.* 1997). Further work demonstrated that this effect is dependent on cGMP and mediated by an increase in the pacemaker current, I_f (Musialek *et al.* 1997; Yoo *et al.* 1998).

In agreement with the findings in isolated hearts or atria preparations, a portion of the positive chronotropic effect of NO donors *in vivo* can occur independently of the autonomic nervous system, as shown by experiments in autonomically denervated and β-blocked rabbits and pigs (Hogan *et al.* 1999*a*; Musialek *et al.* 2000). Importantly, an increase in HR with NO donors has also been reported in healthy human subjects in the absence of changes in arterial BP (Hogan *et al.* 1999*b*) and in heart transplant recipients with cardiac denervation (Chowdhary *et al.* 2002). Similarly, a sustained reduction in HR following chronic NOS inhibition by L-NAME has recently been reported in sino-aortic denervated conscious rabbits (Ramchandra *et al.* 2003). The relative bradycardia observed in conscious eNOS$^{-/-}$ mice suggests that eNOS-derived NO may exert a tonic positive chronotropic action *in vivo* (Shesely *et al.* 1996).

5. THE ROLE OF NITRIC OXIDE IN THE AUTONOMIC CONTROL OF MYOCARDIAL FUNCTION

(a) *Nitric oxide and the response to β-adrenergic stimulation*

A link between β-adrenergic responses and NO has been postulated for many years. Indeed, β-receptor agonists have been shown to stimulate NO production from isolated myocytes (Kanai *et al.* 1997), and a significant body of evidence suggests that the response to β-adrenergic stimulation is potentiated in the presence of NOS inhibitors, both *in vitro* (e.g. Balligand *et al.* 1993; Sterin-Borda *et al.* 1998) and *in vivo* (e.g. Keaney *et al.* 1996). Conversely, NO donors have been shown to attenuate the increase in cell shortening and Ca^{2+} transients by isoprenaline (Stojanovic *et al.* 2001).

Gyurko *et al.* (2000) found that the increase of LV dP/dt_{max} in response to β-adrenergic stimulation was greater in eNOS$^{-/-}$ mice, both in the Langendorff isolated heart and *in vivo*, while overexpression of eNOS depressed the LV response to NA in mouse isolated hearts (Brunner *et al.* 2001). However, an *in vivo* study showed that L-NMMA had no effect on the dobutamine-stimulated increase in LV developed pressure (Hare *et al.* 1995). Interestingly, the HR response to adrenergic stimulation was not altered by either eNOS gene disruption or

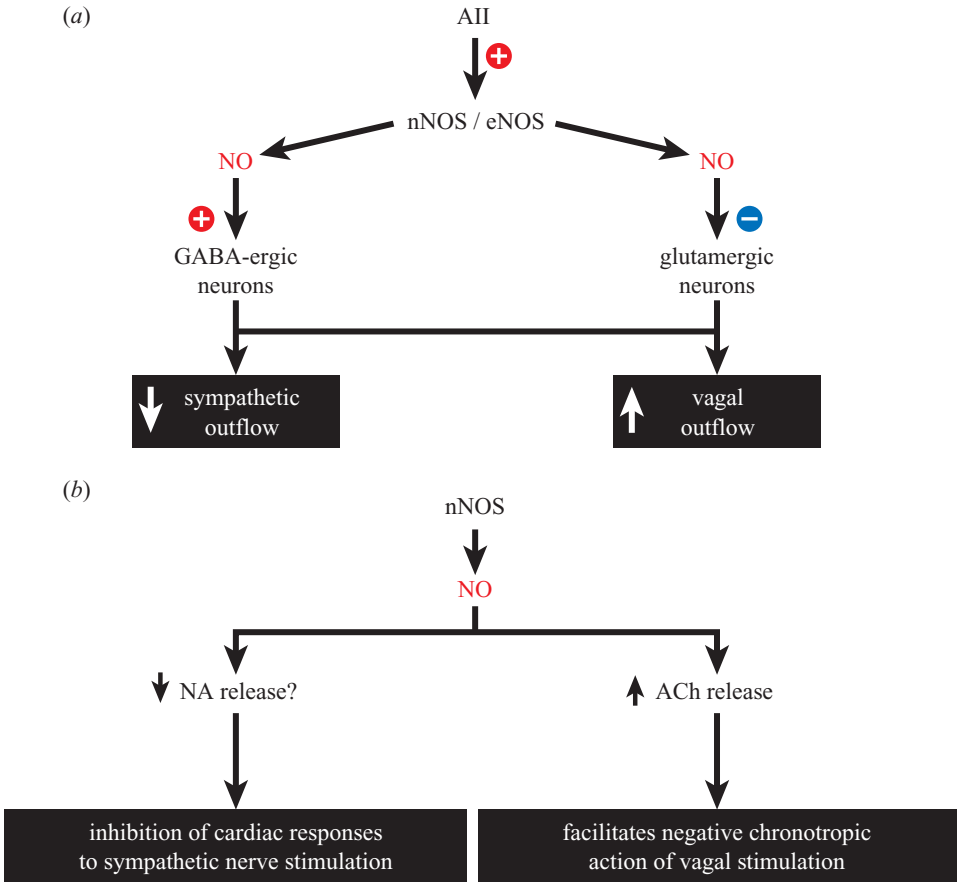

Figure 9. Schematic diagram illustrating the role of nNOS in the autonomic control of cardiac function. (*a*) In the CNS there may be an interaction between the effects of nNOS- and eNOS-derived NO on excitatory and inhibitory inputs in the autonomic control areas. The overall effect of NO appears to be an afferent GABA-ergic inhibition of sympathetic outflow, and an efferent effect of NO to enhance vagal outflow, possibly via glutamergic neurons. This differential control may be brought about by differences in the spatial distribution of the NOS enzymes and localization of their signalling molecules. (*b*) The peripheral actions of nNOS-derived NO decrease NA release (as suggested by Schwarz *et al.* 1995) and facilitate ACh release to inhibit cardiac responses to sympathetic nerve stimulation and to facilitate the negative chronotropic action of vagal stimulation. + (red) denotes a stimulatory action, − (blue) denotes an inhibitory effect.

overexpression (Gyurko *et al.* 2000; Brunner *et al.* 2001). However, in isolated LV myocytes, little effect of eNOS gene disruption has been seen on β-adrenergic responses. For instance, Vandecasteele *et al.* (1999) failed to show any difference in the response of Ca^{2+} current or papillary muscle contraction to adrenergic stimulation in $eNOS^{-/-}$ mice, in agreement with the data of Han *et al.* (1998*a*) and Belevych & Harvey (2000). Interestingly, Godecke *et al.* (2001) found that the dobutamine-stimulated increase in LV pressure was enhanced in isolated hearts from $eNOS^{-/-}$, whereas there was no difference in β-adrenergically stimulated I_{Ca}, indicating that the attenuation of β-adrenergically stimulated contraction by NO is unlikely to occur via inhibition of I_{Ca}. The group that showed an enhanced β-adrenergic response with NOS inhibition in isolated myocytes used L-NMMA added to the internal patch perfusion solution (Balligand *et al.* 1995), and therefore administered direct to the intracellular compartment of the cell, or pre-incubated cells in L-NMMA for a long period (Balligand *et al.* 1993). This illustrates the point made in the introduction that the drugs involved in inhibiting the NOS isoforms must have access to the particular intracellular compartment in which the enzyme is localized for an effect to be seen.

These data and the predominant finding of a depression of the β-adrenergic response by NO when studied in isolated hearts or *in vivo*, compared with the lack of such an effect in isolated myocytes or muscle preparations, could argue that eNOS-derived NO from the endothelium may be essential for this effect to occur in physiological conditions. This idea, however, has not been definitively tested. Additionally, whether NO can been seen to attenuate the β-adrenergic response could be related to level of adrenergic stimulation. For example, in rat ventricular myocytes the effect of an NO donor on spark frequency and Ca^{2+} transients depends upon the degree of adrenergic stimulation and PKA activation (Ziolo *et al.* 2001). Further, the concentration of NO may contribute to the variability (e.g. Ono & Trautwein 1991). Indeed, there are a number of studies showing that NO donors, or addition of components of the NO–cGMP signalling pathway, can inhibit the β-adrenergically stimulated Ca^{2+} current or contraction (e.g. Levi *et al.* 1989; Mery *et al.* 1991; Flesch *et al.* 1997).

In support of a NO-mediated inhibition of β-adrenergic response there is evidence linking eNOS to the downstream signalling pathway of β_3 adrenergic receptors. Unlike the β_1 and β_2 type, β_3 adrenergic receptors are

linked to the G_i protein and their stimulation results in a negative inotropic action (Gauthier *et al.* 1996). β_3 receptors and eNOS co-localize in caveolae (figure 3) (while β_1 receptors seem to be located more abundantly outside these microdomains (Rybin *et al.* 2000)) and it was suggested that stimulation of NO production may be the mechanism underlying their negative inotropic action (Gauthier *et al.* 1998). Varghese *et al.* (2000) showed that β-adrenergically stimulated inotropy was increased in the β_3 knockout mice, as seen in $eNOS^{-/-}$ mice. While NOS inhibition augmented isoprenaline-stimulated contractility in WT mice, this was lost in $\beta_3^{-/-}$ mice. Thus, it appears that the suppression of β-adrenergic inotropy by eNOS-derived NO occurs through activation of the β_3 pathway. In agreement with these findings, Barouch *et al.* (2002) showed that the response to high concentrations of isoprenaline was enhanced in $eNOS^{-/-}$ whereas the selective β_3 agonist BRL37344 had a negative inotropic effect in WT myocytes which was absent in $eNOS^{-/-}$ mice (Barouch *et al.* 2002).

Further light has recently been shed on these complex data concerning the role of endogenous NO in regulating adrenergic responses by studies investigating the effect of nNOS on β-adrenergic inotropy. Our experiments have shown that both the Ca^{2+} transient and the inotropic response to both low (Ashley *et al.* 2002) (figure 7) and high concentrations of isoprenaline (Zhang *et al.* 2003) were increased in LV myocytes from $nNOS^{-/-}$ mice studied at 35 °C, suggesting that nNOS inhibition may account for the increase in β-adrenergic contraction obtained in LV myocytes after non-isoform-specific NOS blockade (Balligand *et al.* 1993). However, Barouch *et al.* (2002) found a depressed inotropic response in $nNOS^{-/-}$ myocytes studied at room temperature. Interestingly, *in vivo* studies from both groups were consistent in showing a depressed β-adrenergic increase in LV systolic function in anaesthetized $nNOS^{-/-}$ mice (Barouch *et al.* 2002; Sears *et al.* 2003*b*). This difference between *in vivo* and *in vitro* results suggests that, as in the $eNOS^{-/-}$ mice, the role of extra-myocardial production of NO (e.g. in the case of nNOS, the contribution of autonomic nerves) may play an important part when studies are conducted in an integrated system.

Thus it seems likely that some synergism exists between the effects of eNOS and nNOS on β-adrenergic stimulation, and differences in this relationship may exist between different experimental conditions or in different species.

(b) *Nitric oxide and sympathetic nerve transmission*

The effects of NO on sympathetic nerve transmission have been reported by several investigators. NA overflow in isolated hearts was increased by NOS inhibition (Schwarz *et al.* 1995), and the NA uptake-1 inhibitor desipramine did not affect this increase. Feelisch *et al.* (1994) reported that in Langendorff rat hearts, exogenously applied NO markedly increased intra-axonal NA levels, suggesting that NO may inhibit NA release. Others reported a reduction in NA uptake with NO donors (Kaye *et al.* 1997). However, when one looks at the effects on sympathetic nerve activity itself, or the resultant effects on the heart, the picture becomes a little clearer. Jimbo *et al.* (1994) showed that a systemic infusion of L-arginine in anaesthetized rabbits caused a decrease in cervical and RSNA. NOS inhibitors enhance the HR response to sympathetic nerve stimulation, while NO donors decrease it, both *in vitro* and *in vivo* (Elvan *et al.* 1997; Sears *et al.* 1998*b*; Choate & Paterson 1999). Data from our group using isolated atria suggest that this is a presynaptic effect of the NO–cGMP pathway, leading to inhibition of transmitter release, as the HR response to bath-applied adrenergic agonists is not affected by NOS inhibition (Sears *et al.* 1998*a*; Choate & Paterson 1999). However, as mentioned previously, data from isolated hearts and *in vivo* studies, where there is presumably a greater release of NO from the endothelium, also support a postsynaptic sympathoinhibitory action of endogenous NO, and thus it seems likely that the pre- and postsynaptic effects work in concert (Balligand *et al.* 1993; Keaney *et al.* 1996; Sterin-Borda *et al.* 1998).

(c) *Nitric oxide and the cholinergic regulation of L-type calcium current and inotropy*

Effects of NO on the cholinergic control of ion currents and inotropy have been shown at the single cell level, predominantly in the presence of prior adrenergic stimulation (known as 'accentuated antagonism'). The NO–cGMP-dependent signalling pathway has been shown to decrease I_{Ca} in β-adrenergically pre-stimulated frog ventricular cells (Méry *et al.* 1993), rabbit sinoatrial node (Han *et al.* 1994) and atrioventricular cells (Han *et al.* 1996) and in mammalian LV myocytes (Levi *et al.* 1989). The muscarinic regulation of acetylcholine-sensitive potassium currents, however, appears to be unaffected by NOS inhibition (Han *et al.* 1994). Balligand *et al.* (1995) showed that NOS or guanylate cyclase inhibitors totally abolished the attenuation of adrenergically stimulated I_{Ca} and inotropy by carbamylcholine (CCh) in rat LV myocytes. Inhibition of I_{Ca} by NO has been shown to occur via PKG in rat and guinea-pig LV myocytes (Mery *et al.* 1991; Mubagwa *et al.* 1993; Wahler & Dollinger 1995) but via cGMP-stimulated phosphodiesterase in frog ventricular myocytes (Méry *et al.* 1993) and in rabbit sinoatrial node cells (Han *et al.* 1998*b*).

A role for NO in accentuated antagonism has also been suggested by *in vivo* and *in vitro* studies. The direct negative inotropic effect of ACh in paced atria was attenuated by NOS inhibition (Sterin-Borda *et al.* 1995), and an *in vivo* study in anaesthetized paced dogs showed that intracoronary infusion of L-NMMA attenuated the vagal antagonism of the effects of dobutamine (Hare *et al.* 1995). However, in this latter study it is possible that the change in coronary perfusion pressure that occurs with L-NMMA may have affected the intracoronary concentration of dobutamine. Further support for the role of NO in the cholinergic control of adrenergically stimulated I_{Ca} and inotropy was shown in $eNOS^{-/-}$ LV myocytes, where there was no significant suppression of isoprenaline-stimulated I_{Ca} or contraction by cholinergic agonists (Han *et al.* 1998*a*).

There are, however, many contradictory data in the literature. In human atrial myocytes there was no effect of NOS or guanylate cyclase inhibition on the effect of ACh on isoprenaline-stimulated I_{Ca} (Vandecasteele *et al.* 1998), and in frog ventricular cells non-isoform-specific NOS

inhibition had no effect on muscarinic inhibition of I_{Ca} following isoprenaline stimulation (Mery *et al.* 1996). Bett *et al.* (2002) contributed to the evidence suggesting that muscarinic-dependent production of NO is not important in the inhibitory actions of ACh on I_{Ca} by showing that CCh-mediated inhibition of I_{Ca} was unaffected by NOS or guanylate cyclase inhibition in ferret right ventricular myocytes, nor was it affected by scavengers of NO. In addition, overexpression of eNOS in mouse hearts failed to affect the response to ACh after noradrenaline stimulation (Brunner *et al.* 2001). Others have shown that the muscarinic antagonism of adrenergically stimulated I_{Ca} and inotropy is preserved in eNOS$^{-/-}$ mice (Vandecasteele *et al.* 1999; Belevych & Harvey 2000; Godecke *et al.* 2001). Several other studies have shown no effect of NOS inhibition (e.g. Stein *et al.* 1993; Hui *et al.* 1995; Kilter *et al.* 1995) on the muscarinic control of LV contractility. Whether this pathway is affected by specific nNOS inhibition, however, has not yet been ascertained.

Further confusion may arise as in some instances there is a clear case for separating the effects of upregulating the NO–cGMP pathway and the functional effects of constitutive NO production in the cholinergic control of the heart. For example, the inhibition of contraction in atrial and ventricular myocardium by cGMP was lost in PKG knockout mice (Wegener *et al.* 2002), but the decrease in inotropy in response to muscarinic agonists was preserved, providing further support for the idea that functionally, at the level of the myocyte, the NO–cGMP pathway is not important in the cholinergic control of contractility. Further, Imai *et al.* (2001) showed that muscarinic agonists led to an increase in cGMP levels via guanylate cyclase in the presence of high levels of cyclic adenosine 3′5′-monophosphate, but blockade of the cGMP-guanylate cyclase pathway did not affect the muscarinic inhibition of I_{Ca}.

It has been suggested that some of the disagreement in the published data concerning the role of eNOS in accentuated antagonism may be a result of the differing ages of the eNOS knockout mice used, and that the LV hypertrophy that develops in these animals during ageing may be responsible for the variable experimental results. Belevych & Harvey (2000) used animals similarly aged to those reported in Han *et al.* (1998*a*) and reported a small but statistically significant increase in cell capacitance (an index of cell size) in eNOS$^{-/-}$ myocytes. Because myocyte size was not evaluated by Han *et al.* (1998*a*), it cannot be categorically excluded that some difference in the degree of LV hypertrophy might have contributed to the difference in their findings. Clearly, whether the development of LV hypertrophy may have some influence on the autonomic control via the NO pathway is worthy of future experimentation.

Another important methodological issue concerns the animals used as controls. In Han *et al.* (1998*a*) sibling controls were used, which would be presumed to be a closer genetic match to the knockout animals than the C57BL/6 or BL6,129 mice used in other studies of eNOS$^{-/-}$ mice (Vandecasteele *et al.* 1999; Belevych & Harvey 2000; Godecke *et al.* 2001). A systematic evaluation of these potentially confounding issues may be helpful in clarifying the controversy.

The point made previously that the use of NO donors may lead to variable results owing to their well-established concentration-dependent effects (reviewed in Casadei & Sears 2003) are also relevant here. Also, there are differences in the NO–cGMP pathway between mammalian versus amphibian species, and results may differ in cells from different regions in the heart (i.e. atria versus ventricles). The effect of NOS inhibitors *in vivo* may be confounded by their effects on BP, blood flow, and on cholinergic and adrenergic nerve transmission, as detailed in subsequent sections. Perhaps in future studies tissue-specific conditional control of NOS gene expression could prove useful in delineating the precise nature and role of NO in the cholinergic control of I_{Ca} and contraction.

(d) *Nitric oxide and the peripheral cholinergic control of heart rate*

There is keen interest in the mechanisms lying behind the autonomic control of the heart. In particular, many studies have focused on the autonomic control of HR, which is of clear clinical relevance, as a decrease in cardiac vagal responsiveness is a powerful predictor of poor prognosis in patients following a myocardial infarction or with heart failure (La Rovere *et al.* 1998; Nolan *et al.* 1998; Cole *et al.* 1999). Vagal stimulation can also help prevent ventricular arrhythmias during exercise and ischaemia in dogs with a healed myocardial infarction (De Ferrari *et al.* 1991; Vanoli *et al.* 1991).

While the cellular studies outlined in the previous section provide some support for the hypothesis that NO may be involved in the cholinergic control of HR (albeit with many contradictory data), up until the late 1990s there were very few studies actually measuring the effect of NO or NOS inhibition on HR itself. In fact, misleadingly, Han *et al.* (1994) claimed an 'obligatory role for NO in the cholinergic control of HR' without having made any measurements of this variable. In cultured spontaneously beating neonatal rat ventricular myocytes, NOS inhibition blocked the negative chronotropic effect of CCh (Balligand *et al.* 1993) and in atrio-ventricular node cells, SIN-1 decreased the frequency of action potentials stimulated by isoprenaline (Han *et al.* 1996). Additionally, in neonatal myocytes from eNOS$^{-/-}$ mice, Feron *et al.* (1998) transfected WT eNOS or an eNOS mutant whose targeting to caveolae had been disrupted. This disruption completely abrogated the negative chronotropic effect of CCh on the spontaneous beating rate, which was restored by transfecting WT eNOS (Feron *et al.* 1998). However, translating such effects on neonatal ventricular myocytes into implications for the autonomic control of HR should be approached with caution. To obtain information on the functional role of NO in the cholinergic control of HR, isolated atrial preparations or *in vivo* studies may prove more useful than investigating isolated myocytes.

Neither NO donors nor cGMP has any effect on the HR response to muscarinic agonists in spontaneously beating isolated atrial preparations (Sears *et al.* 1999; Herring & Paterson 2001). However, NO donors increase the HR response to direct vagal nerve stimulation *in vitro* (Sears *et al.* 1999; Herring & Paterson 2001) and *in vivo* (Sears *et al.* 1999), via a cGMP-dependent (Sears *et al.* 1999; Herring & Paterson 2001) enhanced release of ACh from parasympathetic nerve terminals (Herring & Paterson 2001). These

data indicate that the NO-mediated regulation of cardiac vagal responses occurs at the presynaptic level, although Markos *et al.* (2002) have suggested that NO might also affect vagal transmission at the preganglionic level.

Studies using non-isoform-specific NOS inhibitors have confirmed that the presynaptic regulation of ACh release plays a role in the vagal control of HR *in vitro* and *in vivo*, predominantly in the presence of adrenergic stimulation (Elvan *et al.* 1997; Sears *et al.* 1998*a*; Herring *et al.* 2000). However, studies in the dog and ferret have also shown a role for constitutive NO production in the direct vagal control of HR (Conlon *et al.* 1996; Markos *et al.* 2002).

The NOS isoform involved in autonomic transmission has been identified as nNOS. The decrease in HR with *in vitro* vagal stimulation was slowed in atria from $nNOS^{-/-}$ mice, although the fall in HR was the same in both $nNOS^{-/-}$ and WT (Choate *et al.* 2001; figure 8). In addition, selective inhibition of nNOS causes a reduction in the vagally mediated bradycardia in the ferret, guinea-pig and dog *in vivo* (Conlon & Kidd 1999; Markos *et al.* 2002) and in mouse isolated atrial preparations (Choate *et al.* 2001). That some data have shown little or no role for NO in vagally induced bradycardia (Liu *et al.* 1996; Sears *et al.* 1998*b*) may be related to the developmental stage of the animal, as levels of nNOS in guinea-pig atria have been shown to be increased with age (Herring *et al.* 2000). This phenomenon may also explain the modest contribution of nNOS to the vagal response seen by Choate *et al.* (2001) in relatively young mice (at about three months of age). The finding of a slower HR reduction without a change in the magnitude of the response is strikingly similar to that seen in an earlier paper from our group in young guinea-pig atria (Sears *et al.* 1998*a*), which can be contrasted with the results seen from Herring *et al.* (2000) in older guinea-pigs.

$nNOS^{-/-}$ mice have a higher mean HR than controls and a lower HR variability (Jumrussirikul *et al.* 1998; Choate *et al.* 2001), indicative of a downregulation of cardiac vagal activity. Supportive of this suggestion, the HR response to atropine was much attenuated in the $nNOS^{-/-}$ mice, in contrast to the response to propranolol, which was similar in both groups (Jumrussirikul *et al.* 1998; Choate *et al.* 2001). Thus, it appears that the role of nNOS-derived NO on the vagal control of HR is that of a modulator of the response.

Clearly, the net effect of NO on vagal responses will be the sum of the presynaptic effects and those on the postsynaptic targets, i.e. the cardiac myocytes. At the level of the sino-atrial node the effect of NO on muscarinic stimulation will result from the interplay of its actions on the two pacemaking currents, I_{Ca} (inhibitory) and I_f (stimulatory) (Han *et al.* 1994; Musialek *et al.* 1997). This interplay is illustrated by the fact that inhibition of I_f results in a faster decline in HR with vagal stimulation in isolated guinea-pig atria (Sears *et al.* 1998*a*). At the level of the ventricular myocyte, in the presence of prior adrenergic stimulation, the presynaptic effects of NO to increase ACh release will act in concert with any postsynaptic actions of NO to decrease adrenergically stimulated ion currents or transporters to reduce contractility.

It may be that the modulatory role of NO on cardiac autonomic responses increases in importance in situations in which nNOS is upregulated. A key demonstration of this point was made recently in a study by Danson & Paterson (2003). They showed that exercise training in mice resulted in an enhanced vagal bradycardia, which was inhibited by specific nNOS inhibition, and was associated with a 76% increase in the protein expression of nNOS. The HR response to carbamylcholine did not differ following exercise training, indicative of a presynaptic site of upregulation of the NO pathway (Danson & Paterson 2003). In addition, *in vivo* gene transfer of nNOS augments the HR response to vagal stimulation, thus 'mimicking' the exercise-trained enhancement of vagal function (Mohan *et al.* 2002).

(e) *Nitric oxide and the central autonomic control of the heart*

Afferent inputs from the baroreceptors terminate within the 'autonomic control areas' of the CNS, where the signals are integrated. The NTS receives afferent baroreceptor information via the glossopharyngeal nerve, and output is transferred via the nucleus ambiguus and dorsal motor nucleus to the vagal efferent nerves, and via the caudal ventrolateral medulla and RVLM to the sympathetic efferents. NOS has been found in NTS neurons (Ruggiero *et al.* 1996; Lin *et al.* 2000*a*,*b*), the nucleus ambiguus (Dun *et al.* 1994), in the DMNV (Lin *et al.* 2000*b*), in the ventrolateral medulla (Patel *et al.* 1996) and in vagal afferents (Lin *et al.* 1998*b*).

(f) *Direct effects of nitric oxide on autonomic areas of the central nervous system*

Some investigators have applied NO-modulating agents directly to the autonomic areas of the brain. By using this approach, various studies have shown that NO donors, or the NO precursor L-arginine, in the NTS have a cardiodepressor effect (Jimbo *et al.* 1994; Zanzinger *et al.* 1995*a*; Vitagliano *et al.* 1996; Tseng *et al.* 1996; Lin *et al.* 1999), although Harada *et al.* (1993) found no effect of NTS application of L-arginine on BP, HR or RSNA. Conversely, NTS administration of NOS inhibitors has predominantly been shown to have a cardio-stimulatory effect and to increase RSNA in anaesthetized animals (Harada *et al.* 1993; Tseng *et al.* 1996).

In addition to the effects within the NTS, it has also been shown that NO may act as a sympatho-inhibitory agent within the RVLM (Zanzinger *et al.* 1995*b*). A role for NO in the central parasympathetic control of HR was suggested because application of NO donors within the nucleus ambiguus led to a decrease in HR (Ruggeri *et al.* 2000), while application of NO donors or L-arginine increased the firing rate of DMNV neurons (Travagli & Gillis 1994). Recently, Li *et al.* (2003) showed that nNOS inhibition increased the basal firing activity of PVN of the hypothalamus neurons, but this study did not investigate effects within the intact CNS.

As the majority of these studies have used NO donors or non-isoform-specific NOS inhibitors, further studies are required to pinpoint the exact role and site of action of nNOS within the autonomic areas of the brain.

(g) *Mechanism of action of nitric oxide within the central nervous system*

In the CNS NO has been suggested to inhibit the sympatho-excitatory effects of glutamate (Zanzinger *et al.*

1997), acting in a feedback loop, such that release of glutamate results in activation of NOS, and the resultant release of NO then feeds back to inhibit the subsequent release of glutamate (Garthwaite 1991). Also present in the autonomic control areas are GABA-ergic neurons, and it has been shown that the GABA-ergic system in PVN has a tonic inhibitory effect on the sympathetic nervous system. Zhang & Patel (1998) showed that microinjection of SNP into the PVN caused a reduction in RSNA, BP and HR that was eliminated by blocking the GABA system, while overexpression of nNOS in the PVN resulted in inhibition of RSNA (Li *et al.* 2002), which may involve activation of local GABA-ergic synaptic inputs (Li *et al.* 2003). Thus, an interaction may exist between the effects of NO on excitatory and inhibitory inputs in the autonomic control areas (figure 9). It is conceivable that a differential expression of the NOS isoforms in connection with these excitatory and inhibitory neurons could add further complexity to the central control of the autonomic nervous system. Indeed, nNOS has been co-localized with glutamatergic neurons in the NTS (Lin *et al.* 2000*b*). In addition, as mentioned previously, nNOS is thought to act in a negative feedback manner to inhibit Ca^{2+} entry through the NMDA receptor, with which nNOS co-assembles (figure 6; Kim *et al.* 1999; Baranano & Snyder 2001).

(h) *Nitric oxide and central control of the baroreflex*

As the literature concerning the role of NO in central autonomic baroreflex control contains contradictions and disagreements, it may be useful to bear in mind the following points. First, the use of anaesthetic agents has a confounding effect on the assessment of the arterial baroreflex, and thus perhaps greater weight should be given to studies done in conscious animals. The method of activating the baroreflex may also have a bearing on the results obtained. For example, the rapid rise in BP obtained by bolus injections of vasoconstrictors (i.e. the Oxford method (Smyth *et al.* 1969)) elicits a reflex reduction in HR that is entirely mediated by the vagus, whereas the HR response to sustained BP alterations achieved by infusion of vasoactive agents (i.e. the Korner method (Korner *et al.* 1974)) reflects reflex changes in both vagal and sympathetic activity. These methodological issues may explain why systemic NOS inhibition increased baroreflex gain evaluated by using the Korner technique in conscious rats (Minami *et al.* 1995), while studies employing the Oxford methods have shown either no effect or a reduction in baroreflex gain with NOS inhibitors (Du *et al.* 1992; Jimbo *et al.* 1994; Scrogin *et al.* 1998; Sener & Smith 2001).

Similarly, assessment of baroreflex gain may be influenced by the mode of administration of NOS inhibitors. Sustained changes in BP which accompany chronic NOS blockade may result in baroreflex resetting. Interestingly, however, a recent study in conscious rabbits showed that whereas the baroreflex control of HR reset in response to an AII-mediated sustained increase in BP, sympathetic nerve activity remained suppressed throughout the study (Barrett *et al.* 2003). This elegant work confirmed previous indirect evidence indicating that sympathetic and vagal limbs of the baroreflex may show a different behaviour; hence, the effects of NOS inhibition may vary depending on which effects are studied (e.g. baroreflex control of HR or of renal sympathetic activity). The age of the animals used, as we have noted previously in this review, may also influence the role of NO, as illustrated by the study of Sener & Smith (2001) showing a decreasing influence of NO on baroreflex control with ageing in lambs.

Systemic administration of NOS inhibitors may affect the baroreflex arc at different levels (e.g. it may affect peripheral autonomic transmission, the postsynaptic responsiveness of the target tissue to autonomic stimuli as well as the autonomic circuits in the CNS). Similarly, the complexity and spatial proximity of the central autonomic areas make it difficult to dissect potential site-dependent differences in the role of NO in the regulation of baroreflex responses; this bias may be particularly relevant to studies that have employed high doses of NO donors (or NO precursors) (e.g. Lin *et al.* 1999) or NOS overexpression (Sakai *et al.* 2000).

Finally, some of the differences in the literature may be explained by differing levels of a 'driving factor' for NO production, e.g. AII. For example, Liu *et al.* (1998) saw no effect of L-NAME on sympathetic outflow under basal conditions, but noted an inhibition in the presence of raised concentrations of AII, and Paton *et al.* (2001) showed an effect of NOS inhibition only following AII-induced depression of the baroreflex. As AII levels are raised during anaesthesia, the results of Liu *et al.* may help to explain some of discrepancies between findings in anaesthetized versus conscious animals (Liu *et al.* 1996).

Whether nNOS-derived NO plays a role in baroreflex control in physiological conditions is unclear. Systemic infusion of the relatively nNOS-specific inhibitor 7-nitroindazole had no significant effect on the gain baroreflex control of HR or renal sympathetic activity both in conscious and in anaesthetized rabbits (Murakami *et al.* 1998). Similarly, Paton *et al.* (2001) showed that the AII-induced depression of the baroreflex was unaffected by administration of the nNOS-specific inhibitor, TRIM, in the NTS but was prevented by non-specific inhibition by L-NMMA or by transfection of a dominant-negative mutant of eNOS in rat heart–brainstem preparations (Paton *et al.* 2001; Wong *et al.* 2002). In these experiments, NTS administration of L-arginine or NO donors caused a significant reduction of the baroreceptor reflex bradycardic response, whereas microinjection of NOS inhibitors alone did not affect the gain of the baroreceptor–HR reflex. However, further experiments from the same group showed that eNOS inhibition in the NTS increased the gain of the spontaneous baroreceptor–HR in conscious rats without altering BP (Waki *et al.* 2003). Other studies in conscious chronically instrumented rats did not detect a change in the gain of baroreceptor–HR reflex after inhibition of constitutive NO production or administration of L-arginine in the NTS, although NO increased the HR range during afferent stimulation (Pontieri *et al.* 1998). Both NOS inhibition and application of NO donors in the NTS had no effect on the baroreflex control of RSNA in anaesthetized cats (Zanzinger *et al.* 1995*a*). However, an intra-cerebroventricular application of a sub-pressor dose of L-NAME in conscious rabbits caused an increase in the sensitivity of

the baroreceptor–HR and RSNA reflex (as assessed by the Korner method) (Matsumura *et al.* 1998).

Taken together these data suggest that constitutive release of NO by eNOS in the NTS may inhibit the baroreceptor–HR reflex without affecting the baroreflex control of peripheral resistance.

(i) *Effects of nitric oxide on baroreflex control in humans*

In healthy human subjects we found that systemic SNP administration in the presence of aortic BP clamp had no significant effect on the sensitivity of the baroreceptor–cardiac reflex evaluated both by the phenylephrine bolus technique and by the spontaneous sequence method (Hogan *et al.* 1999*b*). In these subjects, SNP administration was associated with a reduction in the amplitude of the low-frequency component of the diastolic BP variability, suggesting that exogenous NO might decrease the baroreflex/sympathetic control of peripheral resistance. Hansen *et al.* (1994) and Spieker *et al.* (2000) showed no difference in the reduction in muscle sympathetic nerve activity in healthy volunteers after L-NMMA or a phenylephrine infusion titrated to obtain similar finger BP. These findings suggest that endogenous NO production may have little effect on sympathetic nerve activity, although they do not exclude an effect of NO on postsynaptic adrenergic signalling. However, it should be noted that finger BP measurements may not reflect 'central' BP (that is the BP sensed by the arterial baroreceptors) during infusion of vasoactive agents. Specifically, NO donors have been shown to cause a greater reduction in aortic than in peripheral BP (Kelly *et al.* 1990), while L-NMMA infusion in healthy subjects causes an increase in central but not in peripheral pulse pressure (Wilkinson *et al.* 2002). Thus, some caution should be applied when interpreting studies where the effect of NO donors or NOS inhibitors are compared to those of vasoactive agents administered to match the same changes in finger but not in central BP.

In contrast with Hogan *et al.* (1999*b*), other studies have indicated that NO may have a role in promoting tonic and reflex cardiac vagal activity in humans. For instance, stimulation of endogenous NO synthesis by administration of L-arginine caused a reduction in finger BP that (unlike that obtained by hydralazine infusion) was not associated with reflex increase in HR or with a reduction in the amplitude of respiratory sinus arrhythmia and other indexes of cardiac vagal responsiveness (Chowdhary *et al.* 2000). Similarly, a greater preservation of vagal indices was seen in response to SNP infusion compared with hydralazine (Chowdhary *et al.* 2000). Systemic administration of L-NMMA was shown to elicit a smaller increase in HR and other indices of cardiac vagal control than that seen in response to an equipressor infusion of phenylephrine (Chowdhary *et al.* 2000; Spieker *et al.* 2000).

These differences may be related in part to the methodological issues discussed above or to the fact that an increase in cardiac vagal control with exogenous NO administration might have been difficult to detect in the group of young physically trained subjects studied by Hogan *et al.*, who already had a high cardiac vagal 'tone' at baseline. Indeed, the accentuated HR response to vagal nerve stimulation in isolated atria of trained mice has been shown to be mediated by upregulation of nNOS in intracardiac parasympathetic ganglia (Danson & Paterson 2003). Whether this mechanism underlies to the autonomic phenotype induced by physical training *in vivo* remains to be established.

6. IS NEURONAL NITRIC OXIDE SYNTHASE IMPLICATED IN MODELS OF CARDIAC DISEASE?

An increase in cardiac nNOS (Damy *et al.* 2003; Piech *et al.* 2003), eNOS (Khadour *et al.* 1997; Fukuchi *et al.* 1998) and iNOS (Balligand *et al.* 1994; Haywood *et al.* 1996; Lewis *et al.* 1996; Fukuchi *et al.* 1998) expression has been associated with a variety of cardiac pathologies. Increasing interest is being focused upon the potential pharmacological manipulation of NO levels for clinical use (Vallance & Leiper 2002), although NO has been posited as both protective and detrimental to the diseased myocardium. For instance, non-isoform specific inhibition of NOS has been shown to protect against myocardial ischaemia/reperfusion injury in a number of studies (e.g. Patel *et al.* 1993; Schulz & Wambolt 1995), as has the addition of exogenous NO (Bell *et al.* 2003). There is a large literature on the role of iNOS in cardiac disease, but as this has been comprehensively reviewed elsewhere (e.g. Bolli 2001) it will not be discussed here.

(a) *Neuronal nitric oxide synthase in cardiac pathological models*

An increase in myocardial protein levels of nNOS has been shown in postmyocardial infarction in rats, and in spontaneously hypertensive rats (Takimoto *et al.* 2000, 2002; Damy *et al.* 2003; Piech *et al.* 2003). In addition, increased nNOS expression occurs in conjunction with an upregulation of HSP90 and a downregulation of caveolin-1 and 3 (Piech *et al.* 2003). Such changes in HSP90 and caveolins should result in an increase in activity of the NOS enzymes (Venema *et al.* 1997; Garcia-Cardena *et al.* 1998; Bender *et al.* 1999), and therefore, together with the increased NOS expression, they may constitute a compensatory mechanism aimed at maintaining NO bioavailability in the presence of raised oxidative stress with age and left ventricular hypertrophy, as shown by Piech *et al.* (2003) in spontaneously hypertensive rats and by Bryant *et al.* (2002) in a mouse model of hypertrophy. It must be borne in mind that an increase in NOS protein expression may not necessarily correlate with an increase in NO production, as it is becoming increasingly apparent that uncoupling of NOS activity in disease states may lead to NOS synthesis of reactive oxygen species (reviewed in Kojda & Harrison 1999).

Damy *et al.* (2003) and other studies would suggest that upregulation of nNOS may be protective, as inhibition of nNOS worsened LV dysfunction (Song *et al.* 2001*b*; Damy *et al.* 2003). For example, in isolated hearts the depression of LV developed pressure following 40 min of global ischaemia was greater in $nNOS^{-/-}$ than in WT mice (Song *et al.* 2001*b*). In addition, the $nNOS^{-/-}$ hearts developed a larger infarct and a greater expression of tumour necrosis factor α (Song *et al.* 2001*b*). However, in another study of WT and $nNOS^{-/-}$ mice subjected to 20 min of coronary artery occlusion and 120 min of reperfusion, there was no difference in infarct size between the

two groups, although hearts of nNOS$^{-/-}$ mice exhibited more infiltration of polymorphonuclear leucocytes than did hearts of WT mice (Jones *et al.* 2000).

nNOS inhibition with TRIM increased HR significantly more in myocardial infarction rats than in sham-operated rats, suggesting that nNOS upregulation may be protective by enhancing vagal responsiveness after myocardial infarction (Takimoto *et al.* 2002). However, perhaps surprisingly, there was no difference in the response to the muscarinic inhibitor atropine between the two groups (Takimoto *et al.* 2002).

Both protective (Jones *et al.* 1999; Brunner *et al.* 2003) and injurious effects (Wang & Zweier 1996) of eNOS-derived NO on myocardial ischaemia-reperfusion injury have been shown. A finding that could prove to have physiological and clinical implications is that AII increases the protein expression of both nNOS and eNOS in the myocardium (Tambascia *et al.* 2001). However, in hypertensive rats, treatment with an angiotensin-converting enzyme (ACE) inhibitor led to an increase of LV eNOS and a decrease in iNOS expression (Kobayashi *et al.* 1999). We are not aware of a study demonstrating whether ACE inhibition has an effect on myocardial nNOS levels.

On the basis of our previous findings in healthy animals it could be speculated that cardiac upregulation of nNOS in disease states may protect the myocardium from catecholamine toxicity (Ashley *et al.* 2002; Sears *et al.* 2003*b*) and promote a favourable sympatho-vagal balance (e.g. Mohan *et al.* 2002). However, to date, investigations of nNOS in disease models remain limited, for example a study of LV remodelling and survival after myocardial infarction has yet to be carried out in nNOS$^{-/-}$ mice. Given the inhibitory effect of nNOS-derived NO on basal and beta-adrenergic Ca^{2+} fluxes within ventricular myocytes (Sears *et al.* 2003*a*; Zhang *et al.* 2003), and the fact that alterations in Ca^{2+} handling are thought to be a central tenet of myocardial dysfunction (reviewed in Hasenfuss & Pieske 2002), further investigations of the role of nNOS in cardiac disease states may yield interesting findings.

(b) *Neuronal nitric oxide synthase and central autonomic control in pathological models*

Investigations into the effects of NO in the CNS in cardiac disease suggest a role for nNOS. For example, nNOS mRNA (evaluated by semi-quantitative reverse transcriptase–PCR) has been shown to be increased in central autonomic areas of spontaneously hypertensive rats (Plochocka-Zulinska & Krukoff 1997) although constitutive NOS activity was found to be unchanged in these animals as well as in stroke-prone rats (Clavier *et al.* 1994). However, nNOS mRNA level and NADPH-diaphorase-positive cells were significantly reduced in the hypothalamus and brainstem of a rat model of heart failure (Patel *et al.* 1996; Zhang *et al.* 1998). Microinjection of L-NMMA in the PVN of these animals caused a smaller increase in RSNA and BP than in control rats, suggesting that downregulation of nNOS expression in the CNS may contribute to the increase in sympathetic outflow observed in heart failure (Zhang *et al.* 1998). Conversely, nNOS gene transfer into the PVN suppressed RSNA in normal rats (Li *et al.* 2002), and overexpression of nNOS in the RVLM improved the depressed baroreflex function of rats with chronic heart failure (Wang *et al.* 2003).

Further investigations are needed to establish whether the sympathoinhibitory effect of central nNOS overexpression may improve survival in heart failure and other cardiac disease states characterized by neuro-humoral activation and depressed baroreflex sensitivity.

7. CONCLUDING REMARKS

Over the past few years the role of nNOS in the regulation of the cardiovascular system has become more clearly defined. It must now be considered a key component in the effects of NO on the control of cardiac function.

Although there is still much disagreement in the literature, overall it appears that at the myocyte level nNOS-derived NO plays a negative feedback role in inhibiting basal and adrenergically stimulated contraction and Ca^{2+} handling, whereas eNOS may be crucial in mediating the myocardial response to stretch and other mechanical stimuli. In the peripheral autonomic nervous system the picture that is emerging is that nNOS-derived NO plays a presynaptic facilitatory role in cardiac vagal transmission, and may have an inhibitory effect on sympathetic nerve activity.

Postsynaptically, nNOS and eNOS appear to act synergistically to inhibit β-adrenergic signalling and promote cholinergic effects. These effects may be particularly important when NOS expression and activity are driven, for example, as a result of exercise training.

It is becoming increasingly apparent that differences in subcellular localization, mode of action and regulation of constitutive NOS isoforms in the myocardium may subserve their diverse functions and that better understanding of the relationship between NOS enzymes and their effector proteins may help to explain the precise role of NO in regulating cardiac function and in disease. Future experimentation will be needed to elucidate the function of nNOS in myocardial disease. A particularly interesting hypothesis to test is whether nNOS may act as a protective factor in disease, through its inhibitory effect on basal and adrenergically stimulated Ca^{2+} fluxes in the ventricular myocardium, coupled with the vagotonic and sympatholytic effects of nNOS-derived NO in autonomic nerves. However, it is also possible that upregulation of nNOS in cardiac disease states may further reduce adrenergic responsiveness and systolic function. Moreover, in the presence of reduced availability of substrate and cofactors, nNOS could uncouple to produce superoxide, which in turn would lead to impaired NO bioavailability and formation of other potent oxidant molecules such as peroxynitrite. It will only be after some of these questions have been answered that nNOS could be considered as a therapeutic target in cardiovascular disease.

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GLOSSARY

8-br-cGMP: 8-bromoguanosine cyclic 3′, 5′-monophosphate, cGMP analogue
Ach: acetylcholine
AII: angiotensin II
BP: blood pressure
cAMP: cyclic adenosine 3′, 5′-monophosphate
cGMP: cyclic guanosine 3′, 5′-monophosphate
CNS: central nervous system
DMNV: dorsal motor nucleus of the vagus
eNOS or NOS III: endothelial NO synthase
GABA: γ-aminobutyric acid
HR: heart rate
I_{Ca}: L-type calcium current
iNOS or NOS II: inducible NO synthase
L-NAME: N^G-nitro-L-arginine methyl ester, non-isoform-specific NOS inhibitor
L-NMMA: N^G-monomethyl-L-arginine, non-isoform-specific NOS inhibitor
LV: left ventricle
LV dP/dt_{max}: first-order derivative of left ventricular developed pressure
L-VNIO: vinyl-L-N^5-(1-imino-3-butenyl)-L-ornithine
NA: noradrenaline
Na^+–Ca^{2+} exchanger: sodium–calcium exchanger
Na^+–K^+ ATPase: sodium–potassium pump
NMDA: *N*-methyl-D-aspartate
nNOS or NOS I: neuronal NO synthase
$nNOS^{-/-}$: nNOS knockout
$nNOS^{+/+}$: nNOS wild-type
NO: nitric oxide
NOS: nitric oxide synthase
NTS: nucleus tractus solitarii
PKA: protein kinase A
PKG: protein kinase G
PVN: paraventricular nucleus of the hypothalamus
RSNA: renal sympathetic nerve activity
RVLM: rostral ventrolateral medulla
RyR: ryanodine receptor, calcium release channel
SERCA: sarcoplasmic reticulum calcium ATPase, calcium re-uptake pump
SNP: sodium nitroprusside
SR: sarcoplasmic reticulum, cellular calcium store
TRIM: 1-(2-trimethylphenyl) imidazole, specific nNOS inhibitor
WT: wild-type