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Topical Review

Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice

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The modulatory role of endothelial nitric oxide synthase (eNOS) on heart contraction, relaxation and rate is examined in light of recent studies using genetic deletion or overexpression in mice under specific conditions. Unstressed eNOS−/− hearts in basal conditions exhibit a normal inotropic and lusitropic function, with either decreased or unchanged heart rate. Under stimulation with catecholamines, eNOS−/− mice predominantly show a potentiation in their β-adrenergic inotropic and lusitropic responsiveness. A similar phenotype is observed in β3-adrenoceptor deficient mice, pointing to a key role of this receptor subtype for eNOS coupling. The effect of eNOS on the muscarinic cholinergic modulation of cardiac function probably operates in conjunction with other NO-independent mechanisms, the persistence of which may explain the apparent dispensability of this isoform for the effect of acetylcholine in some eNOS−/− mouse strains. eNOS−/− hearts submitted to short term ischaemia–reperfusion exhibit variable alterations in systolic and diastolic function and infarct size, while those submitted to myocardial infarction present a worsened ventricular remodelling, increased 1 month mortality and loss of benefit from ACE inhibitor or angiotensin II type I receptor antagonist therapy. Although non-conditional eNOS gene deletion may engender phenotypic adaptations (e.g. ventricular hypertrophy resulting from chronic hypertension, or upregulation of the other NOS isoforms) potentially confounding the interpretation of comparative studies, the use of eNOS−/− mice has undoubtedly advanced (and will probably continue to improve) our understanding of the complex role of eNOS (in conjunction with the other NOSs) in the regulation of cardiac function. The challenge is now to confirm the emerging paradigms in human cardiac physiology and hopefully translate them into therapy.

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Although the enzymatic capacity of endothelial cells to produce nitric oxide was described as early as 1987 (Palmer *et al.* 1987), the definitive molecular characterization of the endothelial isoform of nitric oxide synthase, or eNOS, was first provided in 1992 (Janssens *et al.* 1992; Lamas *et al.* 1992; Nishida *et al.* 1992; Sessa *et al.* 1992). Analysis of its cDNA confirmed its belonging to a family of three different NO synthases, each encoded by a different gene, i.e. the neural isoform (or nNOS), the inducible isoform (or iNOS, initially cloned from monocytes/macrophages) and the endothelial isoform (eNOS), encoded by *NOS1*, *NOS2* and *NOS3*, respectively. Subsequent expressional studies revealed each isoform to be present in many more cell types than where it was originally discovered, both at the mRNA and at the protein level. Accordingly, eNOS was identified in hippocampal neurons, several epithelial cell types, platelets and cardiac myocytes, in addition to endothelial cells (for a review, see (Forstermann *et al.* 1998). Not unexpectedly, this promiscuity also results in the co-expression of several NOS isoforms within the same cell type, as exemplified in cardiac muscle cells where the constitutive eNOS and nNOS may coexist with iNOS, induced upon stimulation with the appropriate inflammatory mediators. A mitochondrial NOS (mtNOS) has also been identified (Bates *et al.* 1996), corresponding to a variant of neuronal NOS (Kanai *et al.* 2001; Elfering *et al.* 2002), although its functional impact on mitochondrial function *in vivo* remains to be firmly established (French *et al.* 2001).

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Despite this apparent redundancy, the use of isoform-specific inhibitors or genetic deletion experiments has identified a specific modulatory role for each isoform that is subserved by its subcellular localization, at least in the cardiomyocyte. The recent publication of the phenotypic characterization of genetically modified mice in which eNOS is either deleted or overexpressed specifically in cardiomyocytes now motivates the proposal of an updated model of its regulatory functions, almost 10 years after the initial description of the modulation of catecholamine responsiveness of isolated cardiac cells by endogenous NO (Balligand *et al.* 1993).

Molecular regulation of eNOS: an update

Like the other two members of the NOS enzyme family, eNOS contains two functionally distinct domains, i.e. an N-terminal oxygenase, where haeme, tetrahydrobiopterin (BH4) and L-arginine bind, and a C-terminal reductase comprising binding sites for FAD, FMN and NADPH. These two domains are linked by a calmodulin-binding site (aa 499 to 518 of human eNOS) where, upon calcium-induced binding, calmodulin increases the rate of electron transfer from NADPH to the reductase domain flavins and from the reductase domain to the haeme centre for the oxidation of the substrate, L-arginine. Accordingly, inhibition of eNOS activity by calcium removal and calmodulin inhibitors justifies the classification of eNOS (with nNOS) as one of two calcium-sensitive NOSs. Moreover, all NOS function in a dimeric form that is stabilized by haeme and L-arginine, as well as BH4 (Marletta, 1993; Crane *et al.* 1998). Importantly, in the absence of sufficient L-arginine or BH4, 'uncoupled' NOS may generate superoxide anions (O_2^-) instead of NO (Pou *et al.* 1992), leading to the formation of peroxynitrite ($ONOO^-$) resulting from the equimolar reaction of NO and O_2^-. In turn, peroxinitrite may further induce the production of O_2^- by eNOS through oxidation of its zinc–sulphur cluster (Zou *et al.* 2002). The N-terminal domain also contains a glycine residue in position 2 critical for myristoylation, as well as cysteines (Cys15 and Cys26) supporting palmitoylation that are unique to eNOS and importantly condition the enzyme targeting to caveolae (Feron *et al.* 1998*a*). Caveolae are small (70–90 nm in diameter), invaginated foldings of the plasmalemmal membrane that are distinctively enriched in cholesterol and glycosphingolipids, and structurally maintained by oligomerized caveolins that also serve as scaffolds for the assembly of multi-protein signalling complexes within these specialized membrane compartments (Simons & Toomre, 2000). Of note, T-tubule membranes are particularly enriched in caveolae (Levin & Page, 1980) which supports a modulatory role of eNOS in excitation–contraction coupling in the sarcoplasmic reticulum (SR)–T-tubule junctional space (e.g. in response to stretch; Petroff *et al.* 2001), where it may act in concert with nNOS also expressed in SR membranes (Xu *et al.* 1999).

Aside from transcriptional up- and down-regulation (for a review, see Forstermann *et al.* 1998) and the availability of cofactors and substrate (which support eNOS dimeric conformation for optimal catalytic activity), eNOS is also regulated post-translationally by phosphorylation on serine, and, in specific circumstances, also on tyrosine and threonine residues. Stimuli such as insulin or stretch (in cardiomyocytes) (Petroff *et al.* 2001) induce phosphorylation at serine 1177 (for human)/1179 (for bovine eNOS) through PI3K-dependent activation of Akt (protein kinase B), with a subsequent increase in enzyme activity that is less sensitive to fluctuations of intracellular calcium through mechanisms that are presently unclear. Other kinases, e.g. protein kinase A, protein kinase G and AMP-activated kinase also phosphorylate eNOS on serine 1177. AMP-activated kinase and protein kinase C also induce phosphorylation on threonine 495 that inactivates eNOS. According to current models in endothelial cells, stimulation with histamine or bradykinin would first lead to dephosphorylation at this threonine residue, located in the critical calmodulin binding domain, to allow the binding of calmodulin, further stabilized by the subsequent phosphorylation on serine 1177 (Fleming *et al.* 2001).

Regulation of eNOS by protein–protein interactions

Caveolin. eNOS interacts with caveolin-1 (and caveolin-3) in assays of recombinant proteins *in vitro* or in co-immunoprecipitation assays using anti-caveolin-1 antibodies from endothelial cell extracts (and anti-caveolin-3 antibodies in extracts of cardiomyocytes). This interaction both ensures the proper targeting of eNOS (or at least a portion of cellular eNOS) to caveolae and maintains eNOS in an inhibited state. This inhibition can be reversed by addition of exogenous calmodulin, suggesting a reciprocal regulation of the enzyme by inhibitory caveolin *versus* activating calcium–calmodulin (Michel *et al.* 1997). A current model proposes that stimulus- or agonist-induced increases in intracellular calcium promote the displacement of inhibitory caveolin and binding of activated calcium–calmodulin to its consensus sequence on eNOS to initiate catalytic activity. Whether this necessarily implicates translocation of eNOS out of caveolae is still under debate. The phenotypes of mice deficient in either caveolin-1 or caveolin-3 illustrate the functional relevance of the inhibitory 'caveolin clamp' *in vivo*, e.g. vessels from caveolin-1 deficient mice exhibit a marked hyporesponsiveness to constrictor agonists attributable to increased NO release (Drab *et al.* 2001; Razani *et al.* 2001) whereas increased nNOS activity has been observed in skeletal muscle from caveolin-3 deficient mice (Sunada *et al.* 2001) Likewise, our group showed that statins potentiate eNOS activity by decreasing caveolin-1 abundance *in vitro* and *in vivo*, at least in macrovascular endothelial cells where the caveolin pool is lower and the proportion of caveolin-bound eNOS is higher (Feron *et al.* 2001).

The allosteric regulation of eNOS could theoretically be influenced upon stoichiometric changes in the abundance of any of its protein partners that would impact on its binding equilibrium. At one extreme is the total absence of a binding partner, as exemplified in genetic deletion experiments for caveolin-1 and caveolin-3, as mentioned above. Whether the abundance of these proteins changes with pathologic states, especially in heart diseases, has been very little explored. Recently, we showed a reduction of caveolin-1 and -3 (and eNOS) protein abundance in left ventricular tissue of dogs with non-failing, hypertrophic cardiomyopathy induced by perinephritic hypertension (Piech *et al.* 2002*b*) and in similar extracts from spontaneously hypertensive rats (SHR) (Piech *et al.* 2002*a*). Of note, despite reduced eNOS abundance, the tissue levels of cGMP were unchanged, and these animals retained a marked sensitivity to NOS inhibitors, indicating that eNOS catalytic activity was probably maintained through the parallel downregulation of inhibitory caveolins. This emphasizes the need to integrate changes in NOS abundance with those of their allosteric regulators in future studies on cardiovascular diseases to gain further understanding in their functional impact on downstream NO signalling.

Hsp 90. This ubiquitous 90 kDa, heat-shock protein is expressed at high levels (accounting for up to 1–2 % of total cellular protein content) in the cytosol even in unstressed conditions. It functions as a chaperone for the proper folding of specific protein substrates, including many signal transducing molecules (e.g. non-receptor tyrosine kinases, transcription factors and eNOS, among others; for a review, see Richter & Buchner, 2001). Most of its regulatory action in eNOS signalling has been described in endothelial cells. Hsp90 is associated with eNOS in resting endothelial cells and, upon stimulation with vascular endothelial growth factor (VEGF), oestrogen, histamine, shear stress and statins, the association between the two proteins is increased, resulting in enhanced NO production (Garcia-Cardena *et al.* 1998). Of note, the protein kinase Akt (or protein kinase B), the kinase involved in the activating phosphorylation of eNOS on serine 1177, is another client protein for hsp90 and binds to a sequence of hsp90 that does not overlap with those involved in the binding of eNOS. Therefore, hsp90 was recently proposed as an adaptor between Akt and its substrate, eNOS, thereby promoting the activating phosphorylation of eNOS (for more details, see Balligand, 2002).

Modulation of cardiac function by eNOS

We will focus on the specific impact of NO, as released from eNOS, on inotropic, lusitropic and chronotropic aspects of cardiac contraction (for a comprehensive review on all NOSs, see also Massion *et al.* 2001). This will encompass the paradigms deduced from studies in isolated cardiomyocytes, isolated cardiac muscle preparations and *in vivo* assessment of cardiac haemodynamics in animals (mostly in mice with genetic deletion of eNOS, as mentioned in the Introduction), and to a limited extent, in humans. We will distinguish the influence of eNOS in unstimulated cardiac preparations (i.e. in the basal state, without agonist activation) and in stimulated ones (e.g. with catecholamines). In addition, the role of eNOS in unstimulated and stimulated preparations will be compared in 'unstressed' (i.e. in the absence of cardiac injury, such as ischaemia–reperfusion, or infarction) and in 'stressed' preparations.

Role of eNOS in 'unstressed' and 'basal' cardiac preparations

Effect on basal contractility. Some studies previously reported a biphasic inotropic effect of exogenous NO, positive with low concentrations of NO donors but negative with high ones (Kojda *et al.* 1996; Mohan *et al.* 1996; Vila-Petroff *et al.* 1999; Wegener *et al.* 2002). Endogenous NO also may have a positive inotropic effect (Kojda *et al.* 1997; Muller-Strahl *et al.* 2000), as recently evidenced in normal human hearts (Cotton *et al.* 2001). A positive inotropic effect of NO may be explained at the cardiomyocyte level by the potential following mechanisms (see Fig. 1): (1) direct activating nitrosylation of the RyR2 (Xu *et al.* 1998), as demonstrated by nanomolar NO in skeletal muscle (RyR1) (Eu *et al.* 2000) and also probably accounting for the enhanced excitation–contraction coupling gain and positive inotropic effect of cardiomyocyte stretch (Petroff *et al.* 2001); (2) direct *S*-nitrosylation of the voltage-operated L-type calcium channel (VOC) through a redox switch-mediated increase in $I_{Ca,L}$ (Campbell *et al.* 1996); (3) cGMP-independent activation of adenylyl cyclase at low NO levels (Vila-Petroff *et al.* 1999); (4) cGMP-dependent increase in cAMP, through cGMP-mediated inhibition of cAMP PDE III and prevention of cAMP breakdown (Mery *et al.* 1993); (5) PKG-mediated activation of the RyR, through phosphorylation of ADP ribosyl cyclase and cADP-ribose-mediated activation of RyR, as identified in sea urchin eggs (Willmott *et al.* 1996). However, one should bear in mind that the above mechanisms were mostly demonstrated with exogenous NO donors that may not always reproduce the action of endogenous NO released within the boundaries of cellular microdomains. Accordingly, eNOS gene deletion does not influence basal cardiac inotropic state in mice (see Table 1), although this apparently 'neutral' phenotype may result from the confounding effects of several compensatory adaptations. These may include (1) 'backup' production of NO by nNOS, recently shown to exert autocrine modulation of cardiomyocyte function (albeit in divergent fashion – see Barouch *et al.* (2002) and Ashley *et al.* (2002); (2) or iNOS, superinduced in eNOS−/− mice even in unstressed conditions (Sharp *et al.* 2002); (3) production of atrial natriuretic peptide (Gyurko *et al.* 2000). The latter would increase myocardial

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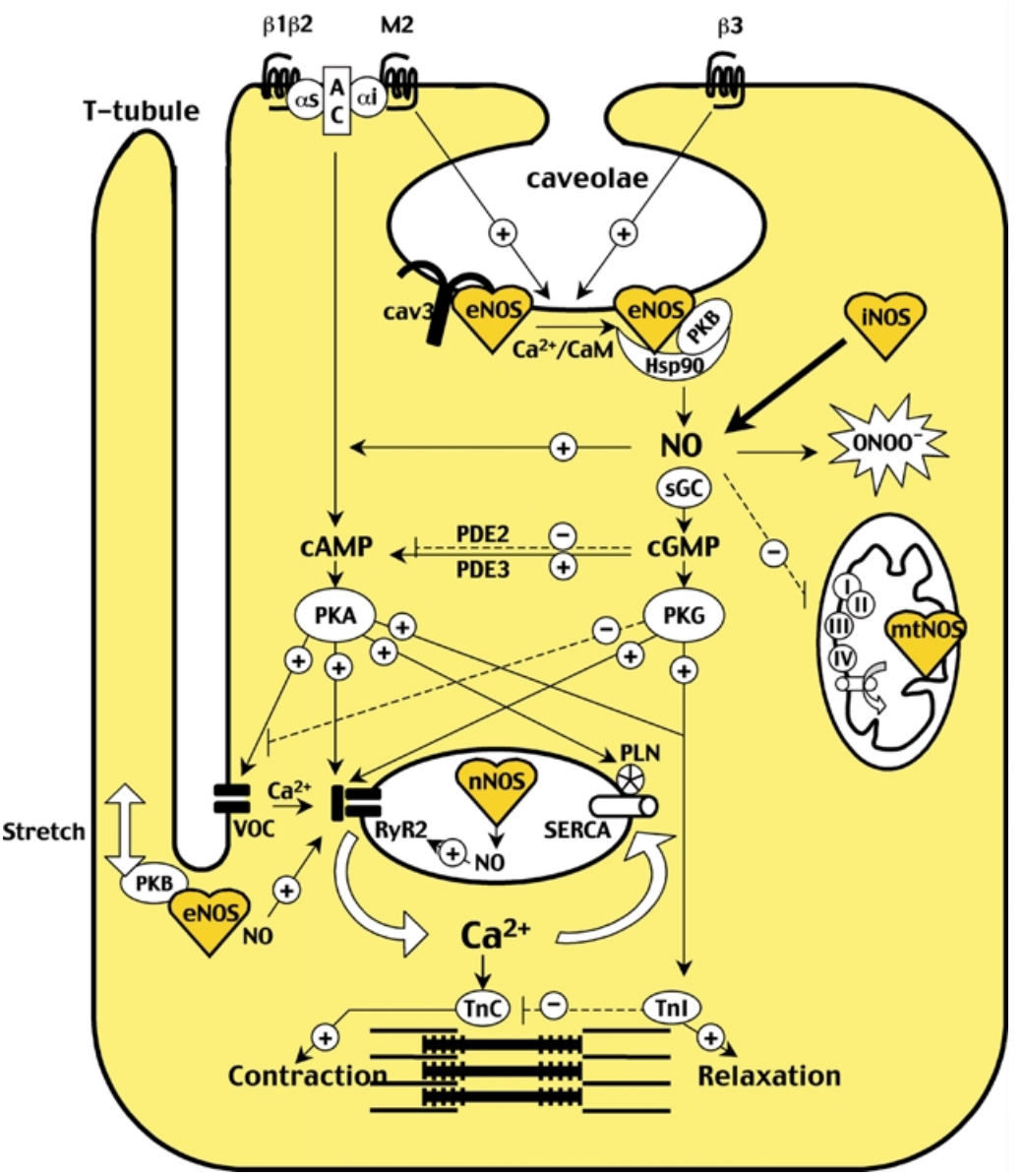

Figure 1. NO signalling pathways in the cardiomyocyte

All NOS isoforms (eNOS, nNOS, iNOS and mtNOS) within specific subcellular compartments of the cardiomyocyte (e.g. sarcolemmal caveolae, SR–T-tubule junction, sarcoplasmic reticulum, mitochondria) are represented, as well as associated regulatory proteins. The net effect of eNOS-derived NO on contractility depends on the specific stimulus (e.g. agonists on β3-adrenoceptor and muscarinic M2 receptor, or stretch), the subcellular compartment (cytosolic or subsarcolemmal) involved and the subsequent amount of NO produced as well as the oxidative status of the cell. On one hand, NO exerts anti-adrenergic inotropic effects after β3-adrenoceptor-dependent activation of eNOS and through cytosolic (and mostly cGMP-dependent) modulation of the main targets of the classical β-adrenergic cAMP–protein kinase A (PKA) pathway, i.e. (1) the voltage-operated L-type Ca^{2+} channel (VOC); (2) the ryanodine receptor Ca^{2+}-release channel type 2 (RyR2); (3) phospholamban (PLN; involved in regulation of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA)); (4) the troponin I (TnI) limiting the sensitivity of troponin C (TnC) to Ca^{2+}. On the other hand, eNOS-derived NO may exert positive inotropic effects, e.g. (1) after sarcolemmal stretch, through subsarcolemmal activation of protein kinase B (PKB), eNOS and probably direct nitrosylation of RyR2 by NO; (2) direct nitrosylation of VOC; (3) direct activation of adenylate cyclase (AC); (4) increase of cAMP after phosphodiesterase 3 (PDE3) inhibition; (5) activation of RyR2 through protein kinase G (PKG)-dependent ADP ribosyl cyclase phosphorylation. αs and αi, G protein subtypes; β1, β2 and β3, adrenoreceptor subtypes; cav3, caveolin 3; CaM, calmodulin; $ONOO^-$, peroxynitrite; I to IV, mitochondrial respiratory complexes; full arrows with + symbol designate stimulation; dashed arrows with – symbol designate inhibition.

Table 1. Inotropic, lusitropic and chronotropic effects of NO in *unstressed* hearts, under basal and β-adrenergic- and/or muscarinic-stimulated conditions

	Inotropic			Lusitropic		Chronotropic		
Model	Basal	β	β+M_2	Basal	β	Basal	β	M_2
eNOS−/−	$=$ [1–12] $\uparrow$ [12]	$\uparrow$ [1–3] $=$ [4] not $\downarrow$ [1]	$=$ [3–4] not $\downarrow$ [5]	$=$ [1, 2, 6–8]	$\uparrow$ [2]	$\uparrow$ [1] $=$ [2, 4, 8, 12, 13] $\downarrow$ [7, 11, 14–18]	$=$ [4]	$=$ [4] not $\downarrow$ [13]
eNOS-OE [19]	$\downarrow$	$=$	$=$	$=$		$=$		

β, beta-adrenergic; M_2, muscarinic type 2; −/−, knockout; OE, over-expressor; $\uparrow$, enhanced effect in genetically modified mice compared with wild-type/control (inotropic, lusitropic or chronotropic, in absolute value); =, similar effect; $\downarrow$, decreased effect; not $\downarrow$, (contractility or heart rate) not decreased by β3 agonist[1] or carbachol[5, 13] in genetically modified mice, while decreased in wild-type. References: 1, Barouch *et al.* 2002; 2, Gyurko *et al.* 2000; 3, Godecke *et al.* 2001; 4, Vandecasteele *et al.* 1999; 5, Han *et al.* 1998; 6, Kanno *et al.* 2000; 7, Yang *et al.* 1999; 8, Scherrer-Crosbie *et al.* 2001; 9, Tada *et al.* 2000; 10, Kubota *et al.* 2000; 11, Liu *et al.* 2002; 12, Sharp *et al.* 2002; 13, Feron *et al.* 1998*b*; 14, Rakhit *et al.* 2001; 15, Sumeray *et al.* 2000; 16, Shesely *et al.* 1996; 17, Godecke *et al.* 1998; 18, Kojda *et al.* 1999; 19, Brunner *et al.* 2001.

cGMP, with effects mostly on relaxation, rather than inotropism (Ji *et al.* 1999). In addition, chronic hypertension in older eNOS−/− mice may induce structural adaptations characteristic of the hypertrophic phenotype, such as reduced arteriolar density (Kubis *et al.* 2002), altered SR calcium load (Boknik *et al.* 2001) or expression of ionic channels (e.g. an increase in $I_{K,ACh}$ (Guo *et al.* 1997), all of which may confound the interpretation of comparative studies with wild-type animals. Conversely, transgenic mice with overexpression of the eNOS gene driven by a cardiomyocyte-specific promoter exhibited a reduction in basal contractile state, probably attributable to the very high level of eNOS expression and deregulated NO production (90-fold higher basal eNOS activity) (Brunner *et al.* 2001). The negative inotropic effect in this case probably involves a desensitization of cardiac contractile myofilaments to calcium.

Effect on basal relaxation. NO exerts a positive lusitropic effect that has been attributed to a cGMP- and PKG-mediated phosphorylation of troponin I, subsequent myofilament calcium desensitization, relaxation hastening and improved distensibility (Shah *et al.* 1994; Layland *et al.* 2002). Indeed, previous studies showed that infusion of intracoronary NO donors in patients induced (1) a relaxation-hastening effect (shorter time to onset of left ventricular (LV) relaxation), secondary (or leading) to abbreviation of contraction (associated with reduced LV end systolic pressure); (2) an improved LV diastolic distensibility (downward shift of the end diastolic pressure–volume relationship, i.e. greater LV end diastolic volume at lower LV end diastolic pressure) (Paulus *et al.* 1994, 1995; Bartunek *et al.* 1997; Paulus, 2001), especially in hypertrophied hearts (Matter *et al.* 1999). The velocity of relaxation ($-dP/dt_{min}$) was not improved in these patients (Paulus *et al.* 1994), contrary to some animal models (Muller-Strahl *et al.* 2000; Hart *et al.* 2001). Notably, the above effects were observed either with exogenous NO donors (*in vivo*, or on isolated cardiomyocytes) or after stimulation with agonists known to activate paracrine NO production from endothelial cells (in whole heart preparations), but the specific implication of unstimulated endothelial or cardiomyocyte eNOS remains uncertain. Analysis of the cardiac phenotype of genetically modified mice revealed that neither eNOS −/− nor cardiomyocyte eNOS-overexpressing mice presented any modification in relaxation under basal conditions *in vivo* (Table 1), which would suggest that eNOS is dispensable for normal diastolic function *in vivo*. The absence of eNOS-derived NO may be compensated by intracardiac production of atrial natriuretic peptide, another stimulant of guanylyl cyclase and cGMP, potentially leading to similar lusitropic properties (Gyurko *et al.* 2000), or NO from residual nNOS with similar positive lusitropic properties, as illustrated in a recent study on nNOS−/− mice (Ashley *et al.* 2002).

Effect on basal heart rate. A distinction must be made here between the involvement of eNOS on spontaneous rhythmicity of pacemaker cells *versus* the modulation by eNOS of the effect of autonomic (i.e. orthosympathetic and parasympathetic) agonists on the heart. Not considered here, but probably relevant to whole organ physiology, is the effect of endogenous NO (from nNOS as well as eNOS) on the control of neuromediator release at the pre-synaptic level, that negatively affects the orthosympathetic input, but reinforces the vagal influence to the heart (Elvan *et al.* 1997; Choate *et al.* 2001). At the cardiomyocyte level, cGMP analogues and NO from endogenous NOS were shown to decrease the spontaneous beating rate of cultured neonatal ventricular myocytes (Balligand *et al.* 1993). Feron *et al.* (1998*b*) subsequently showed the abolition of the response to carbachol in neonatal myocytes from eNOS−/− mice, although the generalizability of this NO dependence for the vagal effect in other cardiac preparations is subject to caution. In

eNOS-deficient mice, baseline heart rate was increased in only one study (Barouch *et al.* 2002), possibly owing to a relative hypovolaemia and/or chronic ventricular remodelling, while the other studies showed normal or decreased heart rate. Since all conscious (unanaesthetised) 2- to 3-month-old eNOS−/− mice (Shesely *et al.* 1996; Kojda *et al.* 1999; Yang *et al.* 1999; Liu *et al.* 2002) presented a decreased heart rate, it is reasonable to assume a positive chronotropic effect of eNOS at the whole organ level. Low concentrations of NO donors were previously shown to increase heart rate *in vitro* (Pabla & Curtis, 1995; Musialek *et al.* 1997), possibly through stimulation of the hyperpolarization-activated inward current (I_f) sensitive to cGMP (Musialek *et al.* 1997, 2000; Yoo *et al.* 1998). The bradycardia observed in eNOS−/− mice may also result from a potential adaptive hyperactivity of nNOS, known to facilitate acetylcholine release at the presynaptic level (Jumrussirikul *et al.* 1998; Choate *et al.* 2001).

Role of eNOS in unstressed but stimulated hearts

Effect on stimulated contractility. The situation is quite different after activation of the β-adrenergic pathway. Under β-adrenergic stimulation *in vivo*, eNOS knockout mice (from two different strains) exhibited a potentiation of their inotropic response (Gyurko *et al.* 2000; Barouch *et al.* 2002). A similar observation was made in isolated mouse hearts from yet another strain, after perfusion with intracoronary dobutamine (Godecke *et al.* 2001). In papillary muscle preparations, another group did not find any difference in the inotropic response to isoproterenol (Vandecasteele *et al.* 1999). Although the apparent discrepancy between paradigms observed in whole organs *versus* papillary muscles may have suggested a significant contribution from paracrine (i.e. endothelial-derived NO) under flow conditions, when contractile shortening was studied in isolated, single cardiomyocytes from eNOS knockout mice, Barouch and colleagues again observed a potentiation of the effects of isoproterenol (Barouch *et al.* 2002), concomitant with an increased calcium transient. This phenotype implies a countervailing effect of autocrine, eNOS-derived NO on the inotropic response to β-adrenergic stimulation, as first suggested almost a decade ago (Balligand *et al.* 1993). Subsequent evidence has indeed demonstrated that β-adrenergic agonists activate a calcium-sensitive NOS in cardiomyocytes (Balligand *et al.* 1995; Kanai *et al.* 1997). Although the molecular mechanism for this activation is incompletely characterized, convergent evidence (Gauthier *et al.* 1998; Moniotte *et al.* 2001) clearly identified the involvement of β3-adrenoceptors for the stimulation of NO production in the human myocardium. Subsequent studies showed that the negative inotropic effect (and decrease in calcium transient) induced by the β3-preferential agonist BRL 37344 is abolished in cardiomyocytes from eNOS−/− mice (Barouch *et al.* 2002), and that the potentiation of the β-adrenergic inotropic effect of NO synthase inhibitors is absent in β3-adrenoceptor knockout mice (Varghese *et al.* 2000), thereby extending the validity of our paradigm in genetically deficient mice.

Once activated, eNOS may counterbalance the adrenergic effect by at least three pathways (see Fig. 1), some of which were identified on the basis of adrenergic inhibition with exogenous NO donors (Mery *et al.* 1993; Campbell *et al.* 1996; Vila-Petroff *et al.* 1999), e.g. (1) cGMP-dependent inhibition of the voltage-operated L-type calcium channel (VOC) and subsequent inhibition of $I_{Ca,L}$ (Campbell *et al.* 1996), either by PKG-dependent phosphorylation of an intermediate protein opposing the effect of PKA (Mery *et al.* 1991) or by increasing cGMP-sensitive cAMP phosphodiesterase (PDE) II activity (Mery *et al.* 1993; Han *et al.* 1998); (2) PKG-dependent phosphorylation of troponin I decreasing troponin C-mediated myofilament responsiveness to calcium (Blumenthal *et al.* 1978; Lincoln & Corbin, 1978), as reproduced with a cGMP analogue (Shah *et al.* 1994), NO donors (Vila-Petroff *et al.* 1999) or NOS stimulation by pacing (Kaye *et al.* 1999); (3) inhibition of RyR2 by NO (Zahradnikova *et al.* 1997), at least after maximal β-adrenergic stimulation (Ziolo *et al.* 2001). Since β-adrenergic-stimulated $I_{Ca,L}$ was unchanged in most experiments using cardiomyocytes from eNOS−/− mice (Vandecasteele *et al.* 1999; Belevych & Harvey, 2000; Godecke *et al.* 2001), the first of the above mechanisms seems unlikely, at least in mouse cardiomyocytes.

Aside from a reduced basal LV-developed pressure, as mentioned above, mice with a large amount of eNOS overexpression (40- to 90-fold, based on enzymatic activity; Brunner *et al.* 2001) exhibited a downward shift of the isoproterenol dose–response curve for left ventricular developed pressure (LVDP) but with unchanged EC_{50}, suggesting that the β-adrenergic effect was superimposed on a constant background production of NO, as if the (vastly) overexpressed eNOS was uncoupled from agonist stimulation in this strain. In contrast, in another strain of transgenic mice with lower amounts of cardiomyocyte-specific eNOS overexpression, the baseline inotropic state of the heart, assessed from LV $+dP/dt_{max}$ *in vivo*, was unchanged, whereas the inotropic response to isoproterenol was attenuated at higher doses of the agonist (S. Janssens, personal communication).

In addition to modulating the positive inotropic effect of β-adrenergic agonists, eNOS was also suggested to mediate, at least in part, the attenuating effect of muscarinic cholinergic stimulation on the β-adrenergic response (Balligand *et al.* 1995; Han *et al.* 1995, 1996; Hare *et al.* 1995), i.e. the classical 'accentuated antagonism' (Levy, 1971). This proposition, however, has been challenged on the basis of subsequent negative studies (Vandecasteele *et al.* 1999; Belevych & Harvey, 2000;

Godecke *et al.* 2001; Bett *et al.* 2002). By contrast, accentuated antagonism was lost in another study using eNOS−/− myocytes (Han *et al.* 1998). Clearly, eNOS is not an obligatory pathway in all species (notably not in frogs) and its implication relative to other signalling mechanisms (e.g. muscarinic cholinergic Gi/o coupling to $I_{K\text{-}Ach}$ or inhibition of adenylyl cyclase) varies at different levels of the heart, i.e. atria *versus* ventricles and pacemaking *versus* working myocytes, which may confound the interpretation of experiments using whole heart preparations. Since these alternative, eNOS-independent pathways (as well as other confounding NO-sensitive currents, such as I_f) are less represented at the ventricular level, this is where the modulatory role of eNOS may be more easily identifiable. Nevertheless, even when using ventricular myocytes, other technical parameters turned out to be crucial for the proper identification of eNOS influence, such as the temperature used for *in vitro* experiments, which is critical for enzymatic activity. Other factors (age-dependent presence of ventricular hypertrophy and potential upregulation of $I_{K\text{-}Ach}$, non-littermate genetic background for control groups) have confounded the interpretation of experiments in eNOS−/− mice that were reported as negative (Vandecasteele *et al.* 1999). A more complete analysis of this contradictory evidence can be found in a previous review on the subject (Balligand, 1999).

Other stimuli than neurotransmitters or hormones may also activate eNOS in the cardiomyocyte. Sarcomere stretching (in the range of physiological elongation, i.e. 12–14 % increase in length) was recently shown to increase eNOS phosphorylation on Ser1179 (consecutive to PI3 kinase activation and phosphorylation of downstream Akt) and induce measurable increases in NO production in single myocytes. This effect was accompanied with an increase in calcium spark rate and a slow increase in calcium transients that was totally absent in cardiomyocytes from eNOS-deficient mice. Notably, these effects were also insensitive to guanylyl cyclase inhibition with1-H-[1,2,4]oxidiazolo[4,3-α]quinoxaline-1-one (ODQ), pointing to a cGMP-independent mechanism, possibly through *S*-nitrosylation of RyR2 (Petroff *et al.* 2001). The relative enrichment of T-tubules in caveolar membranes (where eNOS is localized) would favour a role for eNOS-derived NO in the regulation of EC coupling through its compartmentation in the SR–T-tubule junction, as opposed to its modulation of other aspects of cardiac contraction (as detailed above) that mostly involve increases in cytosolic cGMP. This eNOS-mediated increase in EC coupling gain with stretch could participate in the length-dependent recruitable contractile reserve capacity of the heart, accounting for at least part of the classical Anrep effect.

Effect on stimulated relaxation. Again, the situation may be different under β-adrenergic stimulation, that, by itself, induces a well-known positive lusitropic effect. As for inotropy (see above), β-adrenergic activation of eNOS may similarly oppose the effect of catecholamines on relaxation, instead of being additive. This would be supported by molecular data (Layland *et al.* 2002) showing that troponin I is phosphorylated on the same residues by PKG and PKA, and that the effect of 8-bromo-cGMP to desensitize cardiac myofilaments to calcium was abolished in the presence of isoproterenol (Shah *et al.* 1994) (again arguing for effects of the two interventions that are mutually exclusive). Accordingly, eNOS−/− mice had an improved relaxation under β-adrenergic stimulation (as attested by enhanced $-dP/dt_{min}$ compared to wild-type littermate (Gyurko *et al.* 2000).

Effect on stimulated heart rate. eNOS−/− mice presented no difference in their increase in heart rate under β-adrenergic stimulation compared to controls (Vandecasteele *et al.* 1999), but more inducible ventricular tachycardia after digoxin pretreatment (Rakhit *et al.* 2001) as well as more ouabain-induced arrhythmic contractions and transient inward current (Kubota *et al.* 2000). NO has been shown to transiently increase (Herring *et al.* 2001) or decrease (Yoo *et al.* 1998) I_f during adrenergic stimulation in sinoatrial node pacemaker cells. These studies point to a potential role of eNOS in controlling the sensitivity to arrhythmia that deserves more study in the clinical setting.

Role of eNOS in the stressed heart

Although seminal studies have provided valuable insights into the role of NO in the stressed heart using NOS inhibitors, the relative lack of specificity of these drugs (towards the three NOS isoforms) precludes firm conclusions regarding the specific role of cardiac eNOS. Therefore, emphasis will be put on the latest studies of the phenotype of mice genetically deficient in eNOS submitted to various cardiac insults.

eNOS in acute ischaemia–reperfusion. During ischaemia–reperfusion (I/R), eNOS−/− mice exhibit either improved or decreased contractility, improved or worsened relaxation, unchanged heart rate as well as variable impact on infarct size (see Table 2). Differences in ischaemic (range 16–30 min) or reperfusion times (30–60 min) in the experimental control of coronary flow and heart rate may complicate the interpretation. Studies with the shortest ischaemic time (16 min) at constant coronary flow and pacing (600 min^{-1}) (Flogel *et al.* 1999) showed an improved functional inotropic (LVP), lusitropic and metabolic (phosphocreatine and ATP) recovery, suggesting a detrimental effect of NO in I/R, at least in isolated hearts under these experimental conditions. Possibly, the endogenous production of NO combined with the classical oxidant burst of O_2^- in the initial phase of reperfusion may produce peroxynitrite, a well-known mediator of cellular injury (Beckman *et al.* 1990; Wang &

Table 2. Inotropic, lusitropic and chronotropic effects of NO in *stressed* hearts, under basal conditions, with correspondant infarct size and mortality

Model+stress	Time	Inotropic	Lusitropic	Chronotropic	MI size	Mortality
eNOS−/− +I/R	H0.5–1	↑[1] =[2,4] ↓[4,5]	↑[1,4] ↓[5]	=[2,5–7]	↑[2,3] =[6,8] ↓[3,4] not ↓[6,8]	
+MI	D28–M6	=[9] ↓[10] not ↑[9]	↓[10]	=[10]	=[9,10]	↑[10] =[9]
+apoE−/−[11]	M4	↓	=		↑	

β, beta-adrenergic; −/−, knockout; I/R, ischaemia–reperfusion; MI, myocardial infarction; Time: H, D or M, number of hours, days or months after stress on which parameters are recorded; ↑, enhanced post-ischaemic recovery in genetically deficient mice compared with wild-type/control (better function or relaxation) or enhanced infarct size/mortality; =, similar effect; ↓, decreased effect; not ↓, (MI size) not decreased by ACE inhibitors[6] or early ischaemic preconditioning[8] in genetically deficient mice, while decreased in wild-type; not ↑, (function) not increased by ACE inhibitors[9] nor angiotensin AT1 receptor antagonist[9] in genetically deficient mice, while increased in wild-type. References: 1, Flogel *et al.* 1999; 2, Sumeray *et al.* 2000; 3, Sharp *et al.* 2002; 4, Kanno *et al.* 2000; 5, Hannan *et al.* 2000; 6, Yang *et al.* 1999; 7, Jones *et al.* 1999; 8, Bell & Yellon, 2001; 9, Liu *et al.* 2002; 10, Scherrer-Crosbie *et al.* 2001; 11, Kuhlencordt *et al.* 2001.

Zweier, 1996; Yasmin *et al.* 1997). By contrast, another study (Sumeray *et al.* 2000) in eNOS−/− mice showed an increased infarct size attributable to a permanent decrease in coronary flow, confirming the protective vasodilatory properties of NO. Furthermore, endogenous NO produced by additional NOS isoform induction may exert cardioprotective effects. Accordingly, eNOS−/− mice in the study by Kanno *et al.* (2000) showed a compensatory induction of iNOS after 30 min of ischaemia that resulted in improved function attributed to cardioprotective iNOS-derived NO production. Finally, eNOS−/− mice also lost the benefit from preconditioning with repetitive ischaemic cycles (Bell & Yellon, 2001) and an ACE inhibitor (Yang *et al.* 1999), emphasizing the benefit of NO produced by vascular eNOS in these settings.

The picture may again be different when considering the role of eNOS in the late window of preconditioning (delayed acquisition of tolerance to ischaemia). A substantial body of evidence now supports a critical role for constitutive NOS in the early triggering (Xuan *et al.* 2000) of the induction of a second isoform, or iNOS, that, in turn, ensures a sustained production of cardioprotective NO. Specifically, enhanced NO production by iNOS, moderately and specifically overexpressed in myocytes (Wang *et al.* 2002), is essential to mediate the anti-stunning and anti-infarct actions of late preconditioning elicited by five different stimuli (ischaemia, adenosine A1 agonists, opioid δ1 agonists, endotoxin derivatives and exercise), suggesting that the upregulation of this enzyme is a central mechanism whereby the myocardium protects itself from ischaemia (Guo *et al.* 1999). The molecular and functional aspects of ischaemia-induced late preconditioning can be reproduced by the administration of NO donors in lieu of ischaemia in experimental animals and more recently in patients (Leesar *et al.* 2001), indicating that NO is also sufficient to induce late preconditioning. Accordingly, gene transfer of either eNOS or iNOS has been shown to replicate the infarct-sparing actions of ischaemic preconditioning, suggesting that NOS gene therapy could be an effective strategy for alleviating ischaemia–reperfusion injury (for a complete review, see Bolli, 2001).

eNOS in chronic myocardial infarction. When submitted to coronary ligation, eNOS−/− mice had unchanged infarct size but evident remodelling with decreased capillary density and hypertrophy (unrelated to the development of hypertension), accompanied with subsequent systolic and diastolic dysfunction and increased mortality at 28 days (Scherrer-Crosbie *et al.* 2001). This points to a beneficial effect of eNOS-derived NO on ventricular remodelling after myocardial infarction, as evidenced recently with the eNOS-mediated cardioprotective effects of corticoids (Hafezi-Moghadam *et al.* 2002; Thiemermann, 2002), possibly by increasing coronary collateralization and limiting myocyte hypertrophy. Double eNOS and apoE knockout mice with spontaneous coronary atherosclerosis revealed depressed contractility and increased infarct lesions at 4 months compared with apoE−/− mice (Kuhlencordt *et al.* 2001). A chronically reduced coronary reserve by eNOS ablation may again account for this result. In more chronic post-infarction remodelling conditions (6 months) (Liu *et al.* 2002), no difference has been found between eNOS−/− and wild-type, suggesting an adaptation through compensatory mechanisms (prostacyclin, nNOS,

adaptation of remote myocardium), so that the influence of eNOS in the development and progression of chronic ischaemic heart failure may be considered less prominent, at least in the mouse model. However, the absence of eNOS significantly decreased the long term cardioprotective effects of an ACE inhibitor and an angiotensin II type I receptor antagonist (Yang *et al.* 1999; Liu *et al.* 2002), emphasizing the importance of eNOS-derived NO in modulating the growth/remodelling effects of mediators of the renin–angiotensin system.

Conclusion

The regulatory role of eNOS on various aspects of cardiac contraction, relaxation and rate has become exceedingly complex since its initial identification in cardiac myocytes. The availability of mouse strains with genetic deletion (or overexpression) of specific NOS (e.g. eNOS) allowed a critical re-examination of previous paradigms, including at the whole organ level *in vivo*, both in normal and stressed conditions. It appears that endogenous production of NO by eNOS has little influence on the inotropic or lusitropic state of the heart under basal conditions, but that it consistently opposes the inotropic response to β-adrenergic stimulation. The latter is mediated by eNOS activation through β3-adrenergic receptors, which were identified in cardiomyocytes from several species, including man. This countervailing influence of the β3-adrenergic eNOS pathway on the more classical β1-2-adrenergic positive inotropic effect may protect the heart against the toxicity of excessive catecholamine stimulation. The influence of β3-adrenergically stimulated eNOS must be contrasted with that of cardiomyocyte stretch, which also activates eNOS with a resultant increase in EC coupling gain, calcium transient and contraction force that participates in the length-dependent contractile reserve of the heart. These apparently contrasting effects of eNOS may coordinately influence overall cardiac function depending on the specific stimulus that activates the enzyme and the subcellular compartment where it is activated.

The majority of studies examining the role of eNOS in 'stressed' (mostly ischaemic) hearts conclude that eNOS is protective, both through its vasodilating and anti-thrombotic actions in the coronary vasculature and its critical triggering of iNOS expression and subsequent essential NO production for late preconditioning. Likewise, eNOS opposes the adverse remodelling after myocardial infarction on the short term, but does not influence mortality on longer term, probably owing to alternative compensatory mechanisms. The challenge is now to translate the substantial body of experimental evidence accumulated over the past 10 years into useful therapies by exploiting the beneficial properties of eNOS-derived NO adapted to specific clinical situations.

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