Nitric Oxide and Cardiac Function
Ten Years After, and Continuing

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Abstract—Nitric oxide (NO) is produced from virtually all cell types composing the myocardium and regulates cardiac function through both vascular-dependent and -independent effects. The former include regulation of coronary vessel tone, thrombogenicity, and proliferative and inflammatory properties as well as cellular cross-talk supporting angiogenesis. The latter comprise the direct effects of NO on several aspects of cardiomyocyte contractility, from the fine regulation of excitation-contraction coupling to modulation of (presynaptic and postsynaptic) autonomic signaling and mitochondrial respiration. This multifaceted involvement of NO in cardiac physiology is supported by a tight molecular regulation of the three NO synthases, from cellular spatial confinement to posttranslational allosteric modulation by specific interacting proteins, acting in concert to restrict the influence of NO to a particular intracellular target in a stimulus-specific manner. Loss of this specificity, such as produced on excessive NO delivery from inflammatory cells (or cytokine-stimulated cardiomyocytes themselves), may result in profound cellular disturbances leading to heart failure. Future therapeutic manipulations of cardiac NO synthesis will necessarily draw on additional characterization of the cellular and molecular determinants for the net effect of this versatile radical on the cardiomyocyte biology. **(*Circ Res*. 2003;93:388-398.)**

Key Words: nitric oxide ■ contractile function ■ cardiomyocytes ■ endothelium ■ heart failure

As the prototypical endothelium-derived relaxing factor, nitric oxide (NO) is a primary determinant of blood vessel tone and thrombogenicity. Applied to heart tissue, these functions alone largely justify the growing interest for NO as a regulator of cardiac function. However, the recognition that all three isoforms of nitric oxide synthase (NOS) are expressed in cardiomyocytes themselves has raised several intriguing questions regarding the signaling role of NO in the heart.

The modulatory effects of NO on contractile function are undoubtedly complex.[1–4] Perhaps this is expected when one considers the versatility of NO biochemistry, the multiplicity of its intracellular targets (with sometimes opposite contractile influences), as well as the diversity of its cellular sources within the myocardium. However, subcellular targeting of NO, driven in a stimulus-specific manner, ensures coordinate regulation of cardiac function. Mouse models genetically deficient or overexpressing one or several of the three NOS isoforms helped to clarify the role of endogenously produced NO (versus exogenous NO from pharmacologic sources) in normal or diseased hearts despite several unanswered questions. In the following paragraphs, we attempt to revisit the major paradigms on the influence of NO on several parameters of cardiac contraction with the hindsight of recent knowledge from genetic or molecular characterization of NOS regulation.

Cellular Regulation of NOS

Two major posttranslational modes of regulation of endothelial NOS (eNOS) will be considered here, ie, subcellular targeting and phosphorylation and their operation in the specific context of cardiovascular tissues (for a complete review of the transcriptional or posttranscriptional regulation of eNOS, see the studies by Li and colleagues[5,6]). The regulation of the other isoforms and a summary of the main factors regulating the NOS isoforms in the heart are presented in the online data supplement (including online Table 1), available at http://www.circresaha.org.

Subcellular Location and Scaffold Proteins

The highly reactive nature of NO (a radical gas) mandates the compartmentation of NO synthesis in proximity to its targets for coordinate signaling. Notably, translocation to specific locales is not exclusive to the NOS but also occurs with some downstream effectors, ie, guanylyl cyclase,[7] thereby ensuring efficient confinement of the upstream components of NO signaling. Also, if NO modulates protein activity through the formation of nitrosothiol adducts, anchoring NOS to its target proteins would favor the covalent modification of selective cysteine residues.

eNOS is myristoylated and palmitoylated on glycine (in position 2) and cysteines (in positions 15 and 26), respectively. This double acylation is necessary for the targeting of

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eNOS to plasmalemmal caveolae, as is the interaction with caveolin (caveolin-1 in endothelial cells and caveolin-3 in myocytes[8]), acting not only as a scaffolding protein but also as a negative regulator of eNOS that represses its basal activity. The functional relevance of the caveolin-eNOS interaction was initially established in intact cardiac myocytes[9] and in the endothelium in vivo,[10] where changes in caveolin abundance modulate NO-mediated regulation of beating rate and vascular permeability, respectively. The phenotype of caveolin-1 knockout mice, to some extent, recapitulates these paradigms, because vessels of caveolin-1–deficient animals revealed an increased ability to vasodilate in response to NO-mediated agonists and alterations of the NO-dependent microvascular permeability. Mice deficient in caveolin-3[11] or both caveolin-1 and caveolin-3,[12] on the other hand, develop a hypertrophic cardiomyopathy, although the specific implication of the disruption of eNOS versus other signaling pathways remains undetermined.

Heat shock protein 90, while interacting with eNOS and promoting caveolin-eNOS dissociation, mostly serves as an adapter for the recruitment of other proteins on the complex.[13] Among these is the serine/threonine protein kinase Akt,[14] mediating eNOS phosphorylation on serine 1177 (see below). Association with heat shock protein 90 may also prevent eNOS uncoupling,[15] ie, its production of superoxide anions instead of NO.

Dynamin, another positive regulator of eNOS activity, had previously been identified as a key inducer of caveolae budding and internalization of the muscarinic cholinergic receptor (mAChR)-eNOS complex, the terminating step of mAChR coupling to eNOS in cardiac myocytes[16]; ie, m2AChRs are translocated in caveolae on agonist stimulation,[17] and caveolar location of eNOS is a prerequisite for the NO-dependent m2AChR modulation of cardiac myocyte beating rate (as shown in knockin experiments using wild-type or acylation-deficient eNOS[9]) before the complex is internalized. The interaction between eNOS and caveolin in cardiomyocytes may therefore be viewed not only as a way to restrain basal NO production but also to concentrate the enzyme in discrete locales both to promote its agonist activation and subsequently terminate signaling (the caveolar paradox).[18]

Phosphorylations

The best-characterized residue is serine 1177 (within the eNOS human sequence), which was identified as the target of the protein kinase Akt, itself activated on phosphatidylinositol 3-kinase stimulation.[13] Phosphorylation (and activation) of eNOS on this residue is increased with cardiac muscle stretch and directly correlated with an increased excitation-contraction (EC) coupling gain (see below). Phosphomimetic (S1177D) eNOS was later shown to produce NO even without a maintained increase of $[Ca^{2+}]_i$. Transfection of such constructs in vivo successfully promoted vasoreactivity, angiogenesis, and protection against apoptosis or ischemia/reperfusion.

The pattern of eNOS phosphorylation and dephosphorylation later evolved as exceedingly complex (see the online data supplement). The challenge for future studies will be to examine the relative contribution of each regulatory site on both the level and the time course of NO production. Hopefully, this may help to design smarter eNOS constructs that, on transfection in cardiovascular tissue, would drive NO release where and when required.

NO and Cardiac Contraction: Force-NO Relationship

We will distinguish effects on contracting cardiac preparations either in the absence (baseline) or in the presence of an added inotropic intervention, ie, changes in load or application of inotropic agonists, such as catecholamines (stimulated cardiac preparations). A similar distinction will be made when considering the effects of NO in the normal (unstressed) or diseased (stressed) hearts. The effects of endogenous NO on mitochondrial respiration, apoptosis, and hypoxia sensing[19] as well as on myocardial O_2 consumption, substrate utilization, and myocardial efficiency[20] have been reviewed elsewhere.

Modulation of Basal Cardiac Function by NO in the Normal Heart

Inotropic Effects

In the basal state (as defined above), the effect of NO is bimodal, with a positive inotropic effect at low amounts of NO exposure but a negative one at higher amounts. Several studies also found no effect at all of both exogenous and endogenous NO.[21] Admittedly, defining what low or high amounts really mean is difficult, both in terms of actual quantity of bioactive NO delivered (eg, with different exogenous NO donors) and the correspondence with amounts endogenously produced in vivo. Lack of standardization probably accounts for some of the discrepancy between studies. Also, downstream effects of NO are likely influenced by the interaction with oxidant radicals or scavengers such as myoglobin, particularly abundant in the cardiomyocyte. Accordingly, the effect of NO donors and endogenous NO[22] on contractile force was enhanced in hearts from myoglobin-deficient mice. This buffering effect probably also accounts for the inability to measure extracellular production of NO from unstimulated neonatal or paced adult cardiomyocytes despite their expression of at least two constitutive NOS isoforms. This does not preclude from autocrine NO signaling restricted to microdomains, where cardiomyocyte NOS is localized. Conversely, inotropic effects of endogenous NO have more consistently been observed in beating whole-heart preparations or in vivo, where the stimulation of paracrine NO production from endocardial or endothelial cells by shear or mechanical stress may be at full play.

The bimodal effect of NO (exogenous or endogenous) on cardiac inotropic state is illustrated in the lower curve of Figure 1. With the restrictions as stated above in isolated rat ventricular myocytes,[23] NO delivery from the spontaneous NO donor 2,2-diethyl-1-hydroxy-1-nitroso-hydrazine produced a small (15%) increase in inotropy at submicromolar concentrations. Interestingly, the effect of exogenous NO was potentiated after inhibition of endogenous NO,[24] suggesting that the recruitable positive contractile reserve beyond that achieved by basal NO production in the beating heart is negligible. Although inhibition of NOS may result in a negative inotropic effect, suggesting that

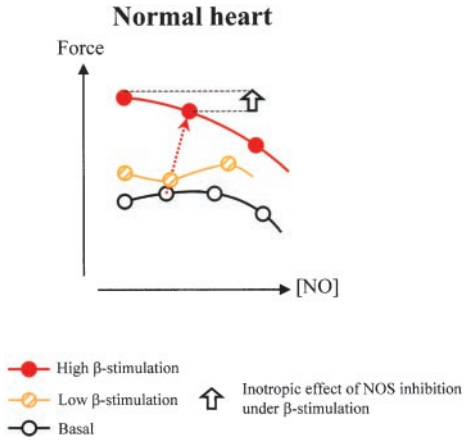

Figure 1. Force-NO relationship in the normal heart. Tentative summary of observed changes in cardiac contraction force as a function of myocardial NO delivery (exogenous or endogenous) in a variety of preparations from normal hearts (see text for details and specific references). The red dotted arrow represents the shift from basal to a β-adrenergically stimulated state, which is accompanied by increased myocardial NO production.

cardiac contraction is constitutively sustained by endogenous NO in some cases, analysis of the phenotype of mice genetically deficient in one or several isoforms of NOS does not uniformly confirm this view (Table 1). Although eNOS$^{-/-}$ mice exhibit normal basal contractility, some[25,26] but not all[27] neuronal NOS$^{-/-}$ (nNOS$^{-/-}$) as well as nNOS$^{-/-}$ plus eNOS$^{-/-}$ mice[27] exhibit increased (not decreased) contractility.

The intracellular mechanisms accounting for the inotropic effects of NO are diverse.[21,28,29] Those mediating the effects of autocrine or paracrine NO are summarized in Figure 2. With exogenous NO, several of these intracellular mechanisms were found to operate in a concentration-dependent bimodal fashion, resulting in inotropically inverse effects at higher NO exposure. In sepsis,[30] large concentrations of NO were proposed to depress cardiac function through cyclic GMP (cGMP)-mediated and protein kinase G (PKG)-mediated desensitization of cardiac myofilaments. A similar mechanism may depress basal cardiac function in mice overexpressing large amounts of eNOS.[31]

Cardiomyocyte nNOS was recently suggested to inhibit basal calcium influx ($I_{Ca,L}$) and contractile shortening, as evidenced from their increase in nNOS$^{-/-}$ myocytes or after acute preferential nNOS inhibition in wild-type myocytes. The dependence of this effect on cGMP remains undetermined.[26] These results are at variance with the absence of changes in basal $I_{Ca,L}$ in nNOS$^{-/-}$ myocytes in another study (also performed at 37°C)[27] or in wild-type myocytes treated with nonspecific NOS inhibitors (at room temperature[32]).

Lusitropic Effects

Desensitization of cardiac myofilaments was also postulated to mediate an increase in diastolic fiber length by NO, as described in isolated cardiomyocytes.[33] At the whole-organ level,[34] this would contribute to the diastolic reserve and also participate in the Frank-Starling mechanism in response to preload increases through enhancement of myocyte distension. Based on endothelium/endocardium disruption experiments, most of the endogenous NO would be produced in a paracrine fashion from endothelial cells. Stimulation of coronary endothelium with NO-releasing agonists such as substance P[34] potentiated the lusitropic effects of NO. Teleologically, this cell to cell cross-talk would provide a way to rapidly adapt myocardial contractility in response to acute changes in preload, perhaps even contributing to compensate altered inotropic properties through increased diastolic reserve in the initial stages of heart failure (HF) (see below). If endothelial eNOS is the main isoform involved, then disruption of its gene in mice would have been expected to result in alteration of diastolic properties. However, this is not apparent from previous studies with NOS inhibitors[35] nor from the analysis of the cardiac phenotype of at least three different eNOS$^{-/-}$ mouse strains (Table 1), including a recent study with full characterization of left ventricular pressure volume relation in vivo.[27] Compensatory production of atrial natriuretic peptide,[36] prostanoids,[37] or NO by myocyte nNOS, acting as a backup lusitropic regulator,[25,26] may be at play. However, the latter would not be reconcilable with the lack of overt diastolic abnormalities in nNOS$^{-/-}$ mice[26,27] (except at very high frequencies[38]), so that endogenous NO-mediated

TABLE 1. Inotropic, Lusitropic, and Chronotropic Effects of NO in Unstressed Hearts, Both at Baseline and After β-Adrenergic- and/or Muscarinic-Cholinergic Stimulation

	Inotropic			Lusitropic		Chronotropic		
Model	Basal	β	β+M_2	Basal	β	Basal	β	M_2
eNOS$^{-/-}$	=/↑	=/↑/*	=/†	=	↑	=/↑/↓	=	=/‡
eNOS-TG	↓	=	=	=	…	=	…	…
iNOS$^{-/-}$	=	=	…	=	=	=	=	…
iNOS-TG	=/↑	…	…	=	…	=/↓	…	…
nNOS$^{-/-}$	=/↑	=/↑/↓	…	=/↓	=	=/↑	…	=/§
n+eNOS$^{-/-}$	=/↑	…	…	↓	…	↑	…	…

β indicates β-adrenergic; M_2, muscarinic type 2; −/−, knockout; TG, transgenic; ↑, enhanced effect in genetically modified mice compared with wild-type/control (in absolute value); =, unchanged; ↓, decreased; * or †, contractility not decreased by β3 agonist* or carbachol† in knockout mice, while decreased in wild-type; ‡ or §, heart rate not decreased by carbachol‡ or vagal nerve stimulation§ in knockout mice, while decreased in wild-type; see references in online data supplement.

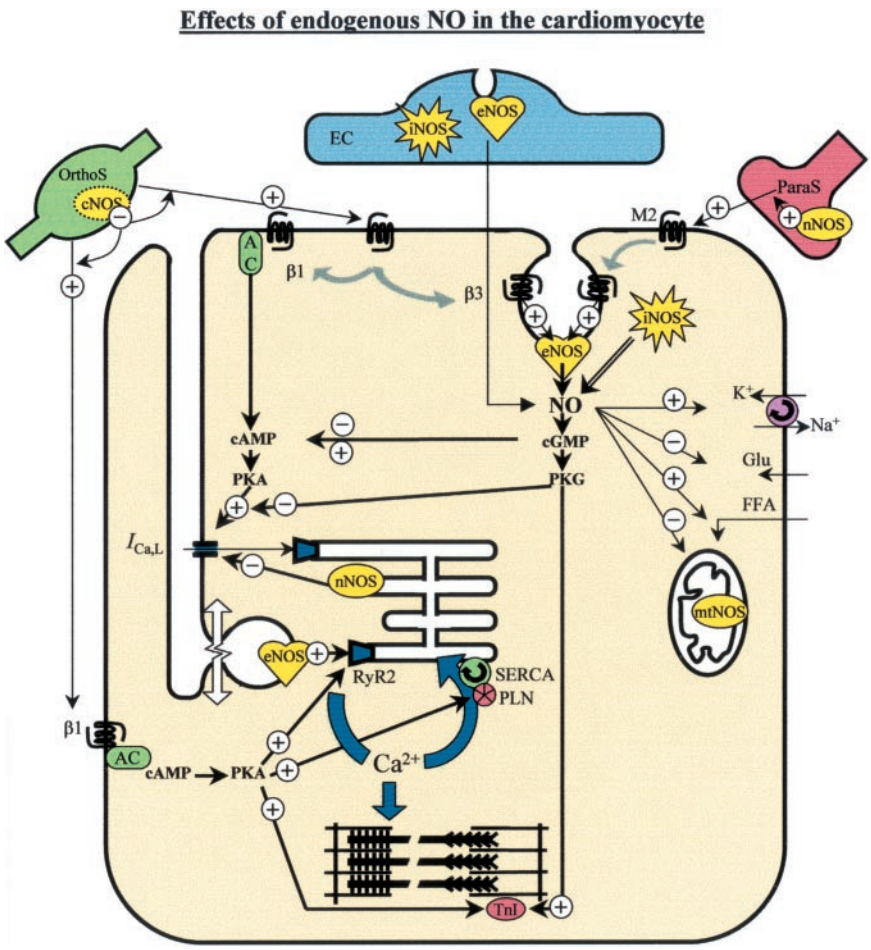

Figure 2. Autocrine and paracrine regulation of cardiomyocyte function by NO. Intracellular effectors of endogenous NO from the three NOS isoforms (eNOS, nNOS, and iNOS) expressed in endothelial cells (EC), sympathetic varicosities (OrthoS), postganglionic parasympathetic fibers (ParaS), or cardiomyocytes themselves. Left, Classical stimulatory effect of β-adrenergic signaling on excitation-contraction coupling, where activation of β_1-adrenoceptors results in adenylate cyclase (AC) activation and subsequent protein kinase A (PKA)-dependent phosphorylation of voltage-operated calcium channels (supporting L-type Ca^{2+} current, $I_{Ca,L}$), ryanodine receptors (RyR2), and phospholamban (PLN) [thereby derepressing the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)] to produce positive inotropic effects, whereas phosphorylation of troponin I (TnI) accounts for positive lusitropic effects. Right, Modulatory effects of endogenous NO on the β-adrenergic pathway. cGMP-dependent mechanisms include phosphodiesterase regulation of cAMP levels, mostly PDEII-induced decreases[46] (exogenous NO may also produce PDEIII-dependent increases in cAMP), and PKG-mediated downregulation of L-type Ca^{2+} currents,[46,115] both of which result in attenuation of β-adrenergic effects. PKG can also phosphorylate TnI to desensitize cardiac myofilaments to Ca^{2+}.[33] These effects have been ascribed both to autocrine NO produced by cardiomyocyte eNOS in response to muscarinic cholinergic receptor (M_2)[47] or β_3-adrenergic receptor[27,58,69] stimulation (although caveolar localization of the latter is not firmly established); or iNOS expressed upon stimulation with inflammatory cytokines[106,116]; and to paracrine NO produced by the same two isoforms in endothelial cells.[28] In addition, nNOS was recently shown to specifically inhibit calcium influx through L-type calcium channels in absence of β-adrenergic stimulation,[26] perhaps providing modulation of excitation-contraction coupling on a beat-to-beat basis. nNOS disruption was also suggested to increase[26] or decrease SR calcium load, eg, upon increases in contraction frequency[38] through undefined mechanisms. NO produced by a constitutive NOS (cNOS) (eNOS or nNOS) in sympathetic varicosities (top left) and parasympathetic neurons (by nNOS; top right) decreases norepinephrine release and potentiates acetylcholine release,[117] respectively, thereby reinforcing antiadrenergic modulation at the presynaptic level. Conversely, localized signaling by eNOS positively regulates excitation-contraction coupling specifically in response to cardiomyocyte stretch (white arrow) independently of cGMP. Finally, other metabolic or ionic effects of endogenous NO are illustrated in the right portion, eg, inhibition of mitochondrial respiration and glucose (Glu) uptake, increase in free fatty acid (FFA) uptake, and activation of Na^+-K^+-ATPase.

lusitropic regulation seems dispensable, at least in the mouse. Mechanistically, it is also unclear how NO-mediated desensitization of cardiac myofilaments would both promote muscle fiber distension and mediate an early increase in force development through the Frank-Starling mechanism, because the latter may itself operate, at least in part, through an increased sensitivity of myofilaments to calcium. However, cardiomyocyte stretch also promotes an autocrine, eNOS-dependent positive regulation of EC coupling and increase in calcium transient[39] that may participate in the late phase of length-dependent activation of cardiac force, as will be detailed later.

Chronotropic Effects

Intracellular increases in cGMP with exogenous and endogenous NO decrease the spontaneous beating rate of neonatal rat or mouse cardiac myocytes.[9,40] The effects of NO on pacemaker cells are more difficult to dissect given simultaneous actions on different targets with opposing effects on their spontaneous depolarization, eg, inhibition of L-type calcium currents but direct activation of the pacemaker current I_f.[41] At the whole-organ level, another control mechanism comes into play through presynaptic modulation of vagal input by nNOS in nerve terminals.[42] Genetic deletion or isoform-specific inhibition of this enzyme has resulted in a decrease of vagal inhibition of heart rate,[43] decrease in its variability, and, under full inhibition of $G\alpha_i$, loss of baroreflex bradycardia.[44] Basal heart rate, on the other hand, is unchanged in most $eNOS^{-/-}$ and some $nNOS^{-/-}$ mice[26,27] (Table 1). Conversely, adenoviral transfection of NOS1 in guinea pig atria potentiated the release of acetylcholine and enhanced the heart rate response to vagal nerve stimulation in vitro and in vivo, whereas the effect of carbamylcholine was unaffected. This strongly supported a facilitating effect of NOS-1 on vagal transmission at the presynaptic level, ie, in cardiac ganglia, where the expression was mostly transduced.[45]

At the postsynaptic level, however, cardiomyocyte eNOS modulates the response to muscarinic cholinergic stimulation.

In atrioventricular node cells, electrophysiological measurements demonstrated a cGMP-dependent inhibition of $I_{Ca,L}$ through phosphodiesterase II (PDEII) degradation of cAMP after β-adrenergic stimulation.[46] The paradigm was initially confirmed in neonatal[9] and adult[47] cardiac myocytes from eNOS$^{-/-}$ mice but subsequently put into question on the basis of other experiments with the same genetic model.[32,48,49] A variety of technical differences, however, may in part explain the negativity of the results from these studies, as commented in more details elsewhere[29] (see also the online data supplement). Also, the expression of the NOS and some downstream NO regulators, such as superoxide dismutase,[50] was shown to vary according to the anatomical origin within the myocardium, so that the relative proportion of subendocardial versus subepicardial myocytes may have contributed to the variability among studies. Finally, the NO-mediated muscarinic cholinergic signaling (and its regulatory role relative to NO-independent mechanisms) was shown to be more prominent in embryonic versus adult heart,[51] raising the intriguing possibility of its resurgence in the diseased heart as part of the reexpression of a more fetal-like gene program.[52] A consensus view would be that nNOS (in cardiac ganglia) and eNOS (in target cardiomyocytes) coordinately reinforce vagal efferent inhibition of heart rate, which would place them among primary candidates for therapeutic manipulation in diseases characterized with loss of parasympathetic heart rate variability.

Modulation of Stimulated Cardiac Function by NO in the Normal Heart

Inotropic Effects

Three stimuli will be considered here, namely, the increase in beating frequency (force-frequency relationship), cardiac fiber stretch, and catecholamine signaling through β-adrenergic receptors, which represent the most common physiological mechanisms to increase cardiac inotropism, eg, during exercise.

Force-Frequency Relationship

In isolated papillary muscles or cardiomyocytes, endogenous NO was shown to contribute a negative inotropic effect, thereby attenuating the positive force-frequency relationship, perhaps through cGMP and PKG-dependent phosphorylation of troponin I and subsequent depression of myofilament response to calcium.[53] Analysis of the force-frequency relationship in nNOS$^{-/-}$ mice demonstrated a role for this isoform in maintaining the sarcoplasmic reticulum (SR) calcium cycling needed for the positive force-frequency response. Accordingly, SR calcium load was decreased with higher pacing frequencies in nNOS$^{-/-}$ cardiomyocytes (whereas it was increased in another set of experiments[26]) through yet undefined mechanisms. NOS3$^{-/-}$ mice had a normal force-frequency response.[38] In healthy human subjects, the positive inotropic effect of increasing pacing frequency seemed unaffected by intracoronary infusion of nonspecific NOS inhibitors.[35] Likewise, the negative force-frequency response and postrest contractile potentiation (both reflecting SR calcium handling) were unaffected by NOS inhibition in rat papillary muscle. In the latter study, NO contributed to the reduction in twitch duration with increased frequency.[54] Together with the NO-induced early onset of relaxation identified in other experiments,[34] this points to a potential role of endogenous NO to regulate the shortening of contraction duration with increased rate.

Cardiac Muscle Stretch

The involvement of endogenous NO in the response of cardiac fibers to stretch was recently demonstrated in isolated rat cardiac trabeculae and single cardiomyocytes, in which the length-dependent increase in Ca^{2+} sparks frequency (as well as whole-cell calcium transient and contraction force) was abrogated by NOS inhibition.[39] Conversely, the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was without effect, suggesting a non–cGMP-mediated mechanism, perhaps through S-nitrosylation of cardiac ryanodine receptors.[55] This, in turn, would increase the EC coupling gain, accounting for the increased sparks frequency in the absence of changes in SR calcium loading or L-type calcium currents. The absence of any effect in single myocytes from eNOS$^{-/-}$ mice demonstrated that sarcomere stretching activated eNOS that was shown to be phosphorylated on S1179 through the phosphoinositide-3-kinase–Akt pathway.[39] This cardiomyocyte eNOS-mediated mechanism may participate in the length-dependent recruitable contractile reserve of the heart, accounting for the slow component of the Frank-Starling response (also known as the Anrep effect). A posteriori, it may also account for the NOS-dependent positive inotropic effect of increases in preload of isolated, perfused hearts, as observed previously.[56]

β-Adrenergic Response

The modulation of β-adrenergic responsiveness by NO has been the focus of intensive investigation after the first demonstration that NOS constitutively expressed in cardiomyocytes attenuated their positive inotropic response to isoproterenol.[40] Almost 10 years later, a large body of (sometimes contradictory) evidence has additionally strengthened this paradigm, albeit with some refinements. As for the influence of NO on basal contractile state, the β-adrenergic inotropic effect can be modulated in a bimodal fashion, depending not only on the concentration of NO but also of catecholamines, as illustrated in Figure 1 (upper two curves). For example, at a fixed concentration of NO, the response to β-adrenergic stimulation was found to be increased at low catecholamines levels but decreased at high levels.[57] However, it should be noted that the potentiation of the β-adrenergic response observed at low concentrations of catecholamines (right portion of the middle curve in Figure 1) has only been evidenced with exogenous NO donors. Conversely, inhibition of endogenous NO resulted in a potentiation of the effect of low[25,40,58–61] or high[62–64] doses of catecholamines in most other studies (left part of the middle and upper curves), and NO (exogenous[65] or endogenous[66]) attenuated the effect of higher doses of catecholamines (right part of the upper curve of Figure 1).

Cardiomyocyte eNOS and nNOS in β-Adrenergic Modulation

The evidence reviewed above supports a role for cardiac NOS as a countervailing mechanism limiting the positive

inotropic, β-adrenergic effect of catecholamines, which then is amplified in cases of NOS abrogation. The discrepancy between the absence of β-adrenergic potentiation in some eNOS$^{-/-}$ preparations, contrasting with increased β-adrenergic response with nonspecific NOS inhibitors in most studies, led to the recent suggestion that another cardiomyocyte isoform, nNOS, may participate in the attenuation of β-adrenergic inotropism.[25] Although one group clearly obtained a potentiation of β-adrenergic response with a nNOS-preferential inhibitor, the paradigm is not uniformly confirmed with nNOS-deficient mice and seems to vary according to catecholamine concentrations.[25,27] At low levels of β-adrenergic stimulation, Ashley et al[25] observed a potentiation of the contractile shortening of cardiomyocytes from nNOS$^{-/-}$ mice, whereas Barouch et al[27] observed a decreased hemodynamic response in nNOS$^{-/-}$ mice in vivo. At high concentrations of catecholamines, nNOS disruption resulted in a decreased shortening (see the online data supplement for a detailed analysis). Conversely, Barouch et al[27] reported a potentiation of the contractile shortening of isolated myocytes at high catecholamines and in vivo indexes of inotropic response (eg, end-systolic elastance) over all ranges of β-adrenergic stimulation in mice genetically deficient in eNOS compared with wild-type controls. This confirms similar findings from others with eNOS$^{-/-}$ mice from a different strain in vivo[36] as well as in isolated perfused hearts (with yet another strain[48]). Transgenic mice with cardiomyocyte-specific overexpression of eNOS also had a downward shift of the dose-response curve of left ventricular (LV) developed pressure in response to isoproterenol.[31] Overall, both transgenic and deficient mouse models would produce phenotypes that are consistent with the proposed regulation of β-adrenergic inotropic response (Figure 1). Subsequent studies may resolve the remaining discrepancy regarding the role of nNOS at low β-adrenergic stimulation. As with all mouse models with nonconditional deletion or overexpression of specific genes, caution should be used regarding the generalizability of these results given the potential confounding effect of chronic compensatory mechanisms.

Coupling of β_3-Adrenoceptors to eNOS

The molecular mechanism for cardiac NOS activation by β-adrenergic stimulation has not been clarified for all constitutive isoforms. As calcium-sensitive enzymes, nNOS and eNOS can be activated on increases in intracellular calcium, eg, after increased pacing frequency[53] or catecholamines. Indeed, β-adrenergic agonists were shown to activate a calcium-sensitive NOS in isolated cardiomyocytes.[40,67] Whether this occurs in cellular microdomains such as caveolae, where β-adrenergic receptors[68] are colocalized with eNOS,[8] or results from broader increases in cytosolic calcium is unknown. Likewise, subsequent posttranslational events regulating cardiomyocyte eNOS activity such as changes in phosphorylation state (eg, on S1177) have only been described in response to stretch,[39] not after agonist stimulation, at least in cardiac muscle cells. Although the involvement of specific β-adrenoceptor subtypes in nNOS activation is unknown, several converging pieces of evidence have identified the critical role of β_3-adrenoceptors for eNOS activation in cardiac muscle from several mammalian species, including humans.[69] Accordingly, inhibition of NOS does not result in the potentiation of the β-adrenergic inotropic effect in mice with targeted disruption of the β_3-adrenoceptor gene,[58] and the NO-mediated negative inotropic effect (and decrease in calcium transient) induced by the β_3-preferential agonist BRL37344 is abrogated in cardiomyocytes from eNOS$^{-/-}$ mice.[27] This β_3-adrenergic eNOS pathway, which is strikingly opposed to the classical positive inotropic effect of β_1-adrenergic (and β_2-adrenergic) signaling, may represent a built-in mechanism of protection against excessive catecholamine stimulation (and downstream oxygen consumption, calcium overload, and toxicity).

Several mechanisms account for the attenuation of NO to the contractile response to β-adrenergic stimulation, as previously reviewed elsewhere[1,21,28,29] (Figure 2). The notion that the major proteins involved in EC coupling (eg, L-type Ca^{2+} channel and ryanodine receptor) are not uniformly distributed along the sarcolemmal membrane[70] supports the concept of specialization of different subsets of cardiomyocyte NOS according to their specific localization, ie, confinement of nNOS in the SR[71] would favor its regulation of calcium-induced calcium release in the dyads; eNOS would modulate the EC coupling gain in T-tubular caveolae close to the SR in response to stretch while regulating the β-adrenergic response in potentially different subsets of caveolae harboring β-adrenoceptors and their downstream effectors. Such compartmentation would also support the differential recruitment of cGMP-dependent versus -independent mechanisms by the same NOS (eg, eNOS), depending on the stimulus (ie, β_3-adrenoceptor activation[69] versus stretch,[39] respectively). Future ultrastructural analysis of NOS colocalization with EC coupling proteins will have to additionally substantiate these interpretations.

Muscarinic Cholinergic Accentuated Antagonism

eNOS activated by muscarinic cholinergic agonists was shown to mediate the classical accentuated antagonism, ie, the ability of muscarinic cholinergic stimulation to attenuate β-adrenergic signaling in various models.[9,46,72,73] This effect probably involved a cGMP-mediated, PDEII-dependent decrease in cAMP.[72] Although the accentuated antagonism was abrogated in isolated ventricular myocytes from eNOS$^{-/-}$ mice,[47] others using either a different strain of mice[48] or the same strain[32] came to opposite conclusions, but under experimental conditions where the relative contribution of NOS versus other muscarinic cholinergic signaling pathways ($I_{K\text{-}ACh}$ or $G\alpha_i$ inhibition of adenylyl cyclase) would seem hardly identifiable. In particular, the latter study[32] is fraught with other confounding variables, eg, lack of proper littermate control mice or use of older mice with significant cardiac hypertrophy, as amply commented elsewhere[21,29,74] (see also the online data supplement).

Lusitropic Effects

In addition to the inotropic effect, NO modulates the lusitropic response to β-adrenergic stimulation. Although the cGMP-mediated desensitization of cardiac myofilaments may predict a positive lusitropic effect additive to that of β-adrenergic stimulation, experiments in isolated cells[75] sug-

TABLE 2. Myocardial NOS Abundance and Activity in the Diseased Heart

	Abundance			Activity			
Disease	nNOS	iNOS	eNOS	Total	nNOS	iNOS	eNOS*
HCM	↑	↑	↓	↓	…	=	…
HF	…	=/↑	↓/=/↑	↑ (early) ↓ (late)	…	↑	↑/=/↓

HCM indicates hypertrophic cardiomyopathy; HF, heart failure; *constitutive NOS (eNOS identity not firmly established); see references in online data supplement.

gested that the PKG-dependent relaxant effect on cardiomyocyte length is absent when the cell has been prestimulated with isoproterenol. Because both PKG and protein kinase A may phosphorylate Troponin I on the same residue, these two pathways may be mutually exclusive to increase lusitropy. Nevertheless, the lusitropic properties of catecholamines were found to be potentiated in eNOS$^{-/-}$ mice[36] (Table 1). Whether this reflects the abrogation of eNOS-mediated antagonism on upstream components of the β-adrenergic pathway (as detailed above) remains undetermined.

Chronotropic Effects

Another interesting benefit of the antagonism of β-adrenergic effect of eNOS on cardiac conduction and excitability is its protection against arrhythmia. Indeed, activation of endogenous NOS was shown to confer increased resistance to ventricular arrhythmia in dogs.[76] Conversely, cardiomyocytes from mice genetically deficient in eNOS displayed a lower threshold for the arrhythmogenic effect of several pharmacologic agents, including ouabain and catecholamines.[77] In whole animals, removal of NOS-mediated control of catecholamine release at sympathetic varicosities may even aggravate the proarrhythmogenic stimulus in addition to loss of direct regulation of the threshold in target cells.

Modulation of Cardiac Function by NO in the Diseased Heart

When trying to transpose the paradigms reviewed above on regulation of cardiac function by NO in the diseased heart, one inevitably recognizes the necessity for nuances according to changes in the expression or activity of each of the NOS isoforms (eg, secondary to alterations in the availability of substrate, cofactors, or allosteric modulators), in NO bioactivity (eg, after increased oxidative degradation), and in vascular or cardiac muscle sensitivity to NO (including from exogenous NO donors), all of which are susceptible to alterations specific to the etiology and stage of the cardiac disease considered. In particular, nonconditional NOS gene deletion (or overexpression) experiments have to be interpreted with caution in mouse HF models. Nevertheless, in the following section, we will review the most recent observations in human and animal models of cardiac diseases (stressed hearts; see also the online data supplement on hypertrophic cardiomyopathy) and will try to build a simplified relationship between NO and cardiac contraction based on emerging paradigms in the failing heart. Changes in myocardial NOS abundance and activity in the diseased heart are presented in Table 2. The involvement of NO in ischemic, septic, and other inflammatory diseases has recently been extensively reviewed elsewhere.[21,78,79]

Heart Failure

Perhaps a simplified representation of the dynamic changes in myocardial NO production with the development of HF is a shift from a spatially and temporally regulated (by eNOS or nNOS) to a deregulated, excessive release (mostly by inducible NOS [iNOS]). An important component of HF is the loss of peripheral and coronary vascular eNOS activity. This is related both to decreased eNOS abundance[80] and a more complex endothelial dysfunction, involving decreased NO bioavailability attributable to increased oxidant stress[81] as well as agonist-specific receptor defects. The reduction in NO-dependent coronary reserve is proportional to the impairment of cardiac function, because the magnitude of coronary blood flow reduction by NOS inhibition is inversely correlated to LV ejection fraction.[82]

Inotropic Effects in the Unstimulated, Failing Heart

A summary of the functional consequences of NOS modulation (or exogenous NO application) on cardiac force, as examined in several models of HF, is tentatively illustrated in Figure 3. Compared with the paradigm in normal myocardium (Figure 1), the curves were shifted downward to account for NO-independent or irreversible processes affecting force development in diseased muscle, among them NO-independent cytotoxic or negative inotropic effects of cyto-

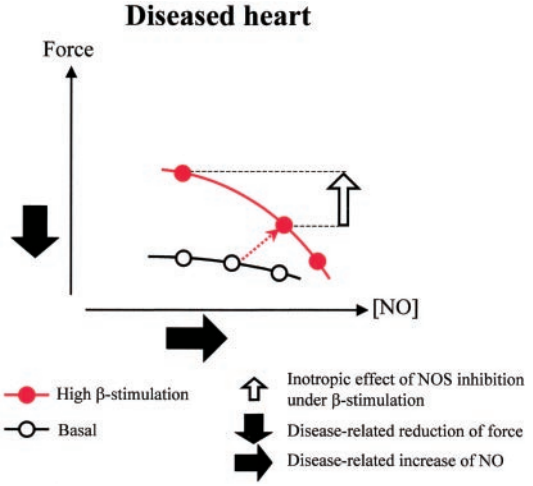

Figure 3. Force-NO relationship in the diseased heart. Tentative summary of observed changes in cardiac contraction force as a function of myocardial NO delivery (exogenous or endogenous) in a variety of preparations from diseased hearts (see text for details and specific references). The red dotted arrow represents the shift from basal to a β-adrenergically stimulated state, which is accompanied with increased myocardial NO production. Note that this shift is accompanied with a higher increase in NO in diseased compared with normal heart (Figure 1).

kines.[83] Similarly, the rightward shift of the curves is a reflection of the increased autocrine or paracrine (including from infiltrating inflammatory cells) production of NO in the failing myocardium, mostly from iNOS. Of note, increased production of NO from residual eNOS may also follow the upregulation of β_3-adrenoceptors in the failing heart.[84]

In most animal models of HF and HF patients, decreasing NO delivery has little, if any, effect on basal (unstimulated) contraction force[21] (Figure 3, lower curve). Likewise, intracoronary infusion of sodium nitroprusside or substance P (to increase paracrine endothelial NO production[66]) has a neutral effect on inotropic indexes in HF patients. Accordingly, in a recent study on mice with cardiac overexpression of tumor necrosis factor-α (TNF-α) (which exhibited increased abundance of iNOS but unchanged eNOS), genetic deletion of iNOS[85] or acute selective iNOS inhibition[86] had no effect on basal contractility indexes in vivo. Cardiomyocyte-specific overexpression of iNOS resulted in little effect on basal contractility[87] but was sufficient to produce cardiomyopathy, arrhythmia, and sudden cardiac death.[88] Of interest, disruption or inhibition of myoglobin was sufficient to induce overt cardiac failure in the context of iNOS overexpression, emphasizing the buffering role of myoglobin on cytoplasmic, iNOS-derived NO.[89,90]

Lusitropic Effects in the Unstimulated, Failing Heart

In HF patients, higher iNOS (and eNOS) mRNA expression (but not proteins) has been correlated with better LV distensibility and preserved LV stroke work.[91] The implication of eNOS in the preservation of LV diastolic properties of the failing heart would be in line with experiments in HF patients infused with enalaprilat (that would activate residual eNOS through bradykinin potentiation).[92] The relationship with iNOS is more disputable in light of other contradictory observations of a negative correlation between iNOS expression and LV ejection fraction (and even diastolic properties)[93] or positive correlation between stable end-products of plasma NO (NOx) and diastolic dysfunction.[94] Also, selective iNOS inhibition[86] or iNOS genetic deletion[85] in TNF-α overexpressing, cardiomyopathic mice had no effect on diastolic parameters. Given the purported cGMP-mediated mechanism for increased ventricle distensibility and relaxation, other mediators known to increase cardiac cGMP levels, such as brain natriuretic peptide, may be more causally related to the preservation of diastolic properties in the failing heart, as directly demonstrated with brain natriuretic peptide receptor antagonists.[95]

Inotropic and Lusitropic Effects in β-Adrenergically Stimulated, Failing Hearts

The blunted response to β-adrenergic stimulation in the failing heart integrates well-established alterations in β-adrenoceptor number or coupling through upregulation of G-protein β-adrenergic receptor kinase (β-ARK) abundance and activity.[96] Along the same lines, our group has identified an alteration in the balance between positively inotropic, β_1- and β_2-adrenoceptors and negatively inotropic, β_3-adrenoceptors in favor of the latter in failing human myocardium.[84] Similar observations were reported in a dog model of HF.[97] Because β_3-adrenoceptors are coupled to NO production (at least in human[69] and murine[58] ventricular tissue), the prevailing β_3-adrenoceptor signaling may participate in the rightward shift to a larger myocardial delivery of NO, as illustrated in Figure 3, for the same amount of β-adrenoceptor stimulation. This may even be reinforced by the fact that β_3-adrenoceptors are more resistant to homologous desensitization, which would support a continuous NO production in the face of the increased adrenergic drive characteristic of HF.[98] In addition to β_3-adrenoceptor coupling to eNOS, a continuous, receptor-independent NO production by iNOS also modulates the inotropic response to catecholamines. Accordingly, NOS inhibition (decreasing myocardial NO) potentiates the β-adrenergic increase in contraction force in several animal models of HF[61,95,99,100] or in HF patients.[101–103] An inverse relationship was also found between iNOS expression or activity and β-adrenergic increase in contraction force in a study of 24 patients with end-stage HF.[104] This confirms our initial paradigms in isolated cardiomyocytes induced with inflammatory cytokines, in which iNOS attenuated the β-adrenergic response.[105,106] Of note, several studies found the potentiation of β-adrenergic inotropic effect with NOS inhibitors to be more pronounced (or exclusively observed) in HF compared with normal hearts (see larger white arrow in Figure 3 compared with Figure 1),[61,85,86,92,100,102,107] a finding not entirely explained by the exclusive expression of iNOS in HF. In the paced dog model, in particular, iNOS is not uniformly detected and eNOS abundance may remain constant.[61,108] One explanation was proposed on the basis of increased caveolin-3 abundance in HF hearts, with increased caveolae density and, possibly, more signaling modules coupling β-adrenoceptors to conserved eNOS proteins. This, however, was not directly measured, nor was the proportion of eNOS interacting with cav-3 directly assayed, eg, in coimmunoprecipitation experiments, an important control to assess eNOS activability, which on the basis of numerous previous studies would be predicted to be lowered (instead of enhanced) in the face of increased cav-3.[9,109] Alternatively, the upregulation of eNOS-coupled β_3-adrenoceptors in this model[97] (as in human HF; see above) would explain both the rightward shift in the dose-response curve for inotropic amines and the higher sensitivity to NOS inhibition. nNOS expression was also found to be increased in the hypertrophic[110] and infarcted heart, where it would mostly reinforce vagal inhibition of heart rate.[111] An increase of paracrine[66,92] or exogenous[104] NO delivery also attenuates the β-adrenergic response, as shown in HF patients from several etiologies.

It would probably be too simplistic to consider the attenuation of the β-adrenergic response by NO as the signature of its major pathogenic role in HF. As mentioned above, deletion of the iNOS gene in transgenic mice with cardiomyocyte expression of TNF-α, despite restoring the contractile response to isoprenaline, did not prevent the development of cardiomyopathy or alterations in contractility indexes. Unlike the β-adrenergic response, the positive force-frequency relationship that is blunted in CHF patients is also insensitive to NOS inhibitors,[35] as is the shortening amplitude of isolated cardiomyocytes from HF patients when increasing pacing rate from 0.2 to 1 Hz.[112] Clearly, additional patho-

genic factors contribute to the late degradation of the contractile performance, as anticipated from early observations in cardiomyocytes.[113] The β_3-adrenoceptor–mediated attenuation of inotropy may even be viewed as a protective mechanism of the failing heart against catecholamine toxicity (at least at initial stages), although this hypothesis still needs rigorous testing in experimental and human HF.[84,98]

Conclusion

Myocardial production of NO is one element in a constellation of physiological regulators of normal cardiac contraction or among the pathogenic mediators of its degradation toward HF. Nevertheless, NO can modulate most other major inotropic interventions and virtually all regulatory steps of EC coupling. In the course of cardiac decompensation, it likely influences several of the central features composing cardiac failure, ie, chamber dilatation, defective β-adrenergic responsiveness, and calcium cycling, leading to altered inotropic and lusitropic properties and arrhythmogenesis.[114] The discovery of the modulation of cardiac contractility by NO has fueled considerable interest and hope in the possibility of reversing cardiac dysfunction with NOS inhibitors. The evidence reviewed above explains why this may not be easily achieved, eg, the influence of endogenous NO is not unidirectional but complex and varies with the etiology and stage of the disease and, foremost, nonspecific NOS inhibition grossly neglects the specialization and spatial confinement of signaling by each NOS isoform both in cardiac muscle and the vasculature. More needs to be learned from isoform-specific, conditional (and perhaps spatially restricted) inhibition or overexpression of NOS in cell and animal models. Future efforts will then have to translate this complexity into more sophisticated therapeutic approaches.

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Expanded Discussion

1. A. Subcellular localization

nNOS. Post- and presynaptic locations of the neuronal NOS is achieved through interaction with different adapters, including the cytoskeletal protein PSD95/93 ([1] and the synapsin/CAPON complex [2]. Although these protein-protein interactions are likely to influence the NO-mediated regulation of cardiovascular function by neuronal cells, the identification of a neuronal-type NOS in the sarcoplasmic reticulum of cardiac myocytes [3] may further extend the functional impact of nNOS compartmentation in cardiac tissue.

iNOS.

Teleologically, the role of iNOS in host defense against injury would not require the fine regulation described above for the calcium-dependent enzymes. Although the regulation of iNOS is largely driven by transcriptional modulation, recent reports have documented the existence of at least four proteins physically interacting with iNOS : kalyrin [4], NAP110 [5],

Rac2 [6] and caveolin-3 [7]. Further characterization is required to evaluate the functional relevance of these interactions in the stressed heart.

eNOS.

The functional impact of the caveolin-eNOS interaction led investigators to formulate the hypothesis that dissociation of the complex might lead to changes in subcellular location. This possibility is strengthened by the recent demonstration that eNOS is a substrate of the acyl-protein thioesterase 1 (APT1) [8]. The activation of APT1 would in part account for the translocation of eNOS from the plasmalemmal pool to intracellular locales upon stimulation by Ca2+-mobilizing agonists, including acetylcholine, bradykinin and estradiol ([9-11]). However, several studies argue against a bulk translocation of eNOS upon its activation [12]. Whether the nature of the agonist (VEGF versus mostly G-protein coupled receptor agonists) accounts for discrepancies between observations is unknown. A consensual view would consider that calcium-mobilizing agonists or shear stress liberate eNOS from its caveolin-1 inhibitory clamp and lead to the formation of new protein multicomplexes. These may include two newly identified eNOS-interacting proteins, NOSIP (for eNOS Interacting Protein) [13] and NOSTRIN (for eNOS Traffic Inducer) [14], both of which were shown to promote the translocation of eNOS from the plasma membrane to intracellular sites, thereby uncoupling eNOS from plasma membrane caveolae and inhibiting NO synthesis.

1B. phosphorylation

nNOS. That nNOS can be phosphorylated by kinases including PKA, PKG, CaMK and PKC has been known for more than 10 years ; only in 1999 was one residue identified as a specific target for CaMK [15]. The functional impact on nNOS-mediated regulation of cardiac contraction, however, remains elusive.

iNOS. No residue has been formally identified within the iNOS sequence as a target for kinases or phosphatases. One study by Pan et al. [16]. documented that the newly synthesized iNOS is rapidly phosphorylated on tyrosine residues in macrophages activated with interferon and lipopolysaccharide. Tyrosine phosphorylation of iNOS may increase NO release.

eNOS. Interestingly, eNOS must be targeted to peripheral (e.g. caveolae) or intracellular membranes to be phosphorylated by Akt [12;17]. The fate of serine 1177-phosphorylated eNOS is however disputed, either translocated *en masse* to the cytosol or moving within membrane structures. However, once released from the caveolin inhibitory clamp and/or any other inhibitory modifications in intracellular membranes, the Ser 1177-phosphorylated enzyme consistently appears more labile and its activity less tightly regulated by its environment. This concept emphasizes the subcellular targeting of the enzyme as a key parameter for both its activation and inactivation. When and for how long, instead of how much, NO is produced appears the critical parameter regulated by phosphorylation changes. This mode of physiological regulation does not preclude the efficiency of phosphomimetic S1177D eNOS mutants in pathophysiological contexts where a net increase in NO production is desirable.

The same serine residue was convincingly identified as a direct substrate for other kinases including the AMP-activated kinase [18], PKA, PKG [19] and the CaM-dependent kinase II [20]. Interestingly, cardiac ischemia was shown to stimulate phosphorylation of eNOS by AMPK [18], thereby providing a direct link between metabolic stress and heart function. Although the many identified kinases leading to Ser1177 eNOS phosphorylation and the *in vivo* effects of the S1177D phosphomimetic support the functional role of this phosphorylation, the interpretation of the data is potentially confounded by changes in other phosphorylation sites within the eNOS sequence. Indeed, four other phosphorylatable eNOS

residues have been identified, e.g. Ser116, Thr495, Ser 615 and Ser 633, all involved in changes in the enzyme activity. Phosphorylation of Thr495 by the AMPK reduces eNOS activity [18]. Notably, changes in phosphorylation of these residues follows exposure to the same agonists which induce Ser 1177 phosphorylation. Accordingly, bradykinin and histamine stimulation of endothelial cells leads to both Ser1177 phosphorylation (through the CaMKII) and Thr495 dephosphorylation [20;21] to coordinately activate eNOS. Moreover, important differences exist between the phosphatases activated by agonists to modulate eNOS activity. For instance, the phosphatase PP2A promotes Ser1177 dephosphorylation[22] whereas VEGF dephosphorylates Ser116 through calcineurin (PP2B)-mediated pathway[23]. Dephosphorylation of Thr495 can be produced both by PP1 and PP2B (in a mutually exclusive manner) in response to various eNOS-activating agonists [20-23].

The Ser615 and Ser633 phosphorylations are less well characterized but were recently reported to be mediated by Akt and PKA, respectively. The former was proposed to reduce the Ca-CaM dependency of eNOS activity, whereas the latter would lead to a direct increase in maximal activity. Interestingly, Ser633 is located in a defined autoinhibitory domain within the flavin-binding of eNOS and its phosphorylation by PKA and PKG is thought to facilitate the displacement of this domain and to promote eNOS activation [19].

2. Muscarinic cholinergic inhibition of heart rate

Vandecasteele et al ([24]) used inapproriate, non-littermate control animals ; studied older (3-6 months) mice with significant hypertrophy, a confounding phenotype potentially associated with independent changes in NO-independent receptor function and coupling (e.g. IKAch ; [25]) ; performed single myocyte studies at room temperature, which, in addition to specific aspects of EC coupling (cADPR [26]) may affect NOS function and its contribution to the endpoints studied relative to other coupling mechanisms (e.g. IK-Ach and inhibition of adenylyl cyclase). Others [27;28] later obtained similar negative results ; Belevych et al [27] used

isolated cells from younger (2-4 months) animals from the same strain (and littermate controls) at 32°C but with a positive index of hypertrophy (measured from membrane capacitance) and studied only Ica-L (which is only one aspect of contraction) ; Godecke et al [28] studied both Ica-L and contraction at 32°C in isolated cells and hearts from a different strain, but used older (3-6 months) mice. Also, they found no attenuating effect of a cGMP analog on Ica-L at baseline in eNOS-/- cells (contrary to wild-type cells), whereas beta-adrenergically-stimulated cells did respond ; this may suggest abnormal responsiveness of L-type calcium channels to cGMP (perhaps as part of the hypertrophic phenotype), whereas upstream steps of beta-adrenergic signaling retained sensitivity (e.g. adenylyl cyclase, cAMP and PKA), explaining the attenuation of adrenergically-stimulated currents. Neither provided any independent assessment of NOS function in their experimental conditions, whereas Han et al [29] verified consistent changes in cGMP levels. Therefore, the persistence of rate decrease or accentuated antagonism in eNOS-/- cells may well be explained by alternative cholinergic regulation of upstream beta-adrenergic signaling (e.g. adenylyl cyclase inhibition) in conditions where eNOS (in control cells) is inactive and/or the target (L-type current) is insensitive to NO/cGMP (in hypertrophic eNOS-/- cells).

3. nNOS regulation of beta-adrenergic signaling.

Ashley et al [30] used pacing frequencies from 1 to 6 Hz and a single, low concentration of Iso (2nM, corresponding to the EC50 in their hands), whereas Barouch et al [31] used 1 Hz and constructed a full dose-response curve. In fact, both studies identify a neutral effect of NOS1 disruption at nanomolar concentrations of Iso and 1 Hz. Although it may be argued that higher pacing frequencies (and 37°C, as opposed to room temperature) may be closer to physiologic conditions in the former, a full characterization of contractility indexes from pressure-volume loops in vivo in the latter confirmed that nNOS disruption resulted in a

blunted (not potentiated) inotropic response to Iso infusions. At high concentrations of catecholamines, the inotropic response is suppressed both in isolated cardiomyocytes and in vivo. Double eNOS/nNOS -/- mice had a response to Iso that was not different from wild-type controls [31].

4. Hypertrophic cardiomyopathy

The signaling pathways mediating physical stress and/or agonist-induced activation of the genetic program leading to cardiomyocyte hypertrophy are susceptible to modulation by exogenous or endogenous NO at various levels. In addition to NO's ability to regulate the release of paracrine growth factors (such as endothelin or norepinephrine -see above), the interference of NO or cGMP in the intracellular cascade(s) mediating cardiomyocyte hypertrophy has received much attention, both in cultured cells in vitro [32]and in vivo [33]. Recently, cGMP-dependent protein kinase (PKG) was shown to interrupt the calcineurin and NFAT –dependent hypertrophic response to α1-adrenergic stimulation, probably through PKG attenuation of L-type calcium currents [34]. Additional effects, e.g. scavenging of oxidant radicals by NO may be at play, since α1-adrenergic induced hypertrophy was also shown to implicate NADPH oxidase activation, ROS generation and Ras-MEK1/2-ERK1/2 (mitogen- and extracellular signal-regulated kinase) activation [35]. Integration of these effects at the whole cell or organ level may be complex, given additional targets for NO e.g. on mitochondrial function, as reviewed above. Indeed , mitochondria isolated from hypertrophic cardiomyocytes are more sensitive to inhibition by NO which may be more abundantly released from iNOS in the hearts of aorta banded rats [36]. This may result in more inhibition of mitochondrial respiration especially under stress [36]. An intriguing hypothesis would be that uncontrolled, sustained NO production and mitochondrial inhibition under hypertrophic stimuli would favor the transition from compensated to decompensated hypertrophy through

pro-apoptotic effects of NO (see above), as part of a shift of balance toward increased apoptosis [37;38]. Again, the local redox milieu, NOS isoform involved and amount of bioactive NO released will presumably dictate the final effect.

Few studies have examined the impact of NOS isoforms deletion/overexpression on the development of hypertrophic cardiomyopathy. In one strain of mice with moderate eNOS overexpression under the control of the cardiomyocyte-specific α-MHC promoter, indexes of myocardial hypertrophy were reduced in response to aortic banding. A similar benefit was reported after chronic isoproterenol infusion in another strain with endothelium-specific eNOS overexpression, that was at least in part attributable to a blood pressure lowering effect [39]. Conversely, NOS1 and NOS3-/- mice develop cardiac hypertrophy by the age of 5 months [31]. Although the hypertensive phenotype of NOS3-/- mice may in part explain the development of hypertrophy, NOS1-/- mice do so despite maintained normal blood pressure. Double NOS1/NOS3-/- mice also develop a higher degree of hypertrophy than NOS3-/- despite similar blood pressure levels.

The effects of NOS inhibition on inotropic indexes in whole heart or in vivo are difficult to interpret in the context of overt hypertrophic cardiomyopathy, in part due to the coexistence of decreased capillary density and endothelial dysfunction lowering the threshold for ischemia. In addition , the sensitivity of hypertrophic cardiomyocytes to endogenous or exogenous NO may be altered [40]. These and other mechanisms may explain the neutral effect of NO on inotropic indexes (dP/dtmax) in aortic-banded guinea pigs hearts, even in the presence of antioxidants [41]. Hypertrophic hearts typically display altered basal LV relaxation [42] which may be critically dependent of residual calcium-dependent NOS activity [43]. Some of this impaired relaxation has been attributed to the inactivation of endothelium-derived NO by oxidant radicals (perhaps produced by NADPH oxidase, the expression and activity of which are increased in hypertrophic hearts)[41]. Accordingly, the relaxation-hastening effect of NO

was restored by antioxidants and compensated for upon infusion of exogenous NO donors [44]. The latter were also shown to improve relaxation in patients with hypertrophic cardiomyopathy secondary to severe aortic stenosis [45]. Among drugs currently used to treat cardiovascular diseases, those endowed with the ability to enhance endogenous NO production such as angiotensin converting enzyme inhibitors or statins may similarly improve cardiac function through NO-dependent lusitropic properties.

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Online Table 1. Differential regulation of NOSs in the heart.

	NOS abundance		NOS activity
nNOS			
+/0	Chronic angiotensin II [1,2]	+	Hsp90 [54,55]
–	Chronic intermittent hypoxia [3]	**–**	dystrophin deletion (mdx mice)[56]
iNOS			
+	IL1β, INFγ[4,5-7] LPS, TNFα+IL6[6,8,9] Phenylephrine (α-AR) [10,11] Norepinephrine(α and β) [12] Isoproterenol (β2) [13] Hypoxia [14] High glucose[15] C-reactive protein[16] Estrogen [17]		
–	Corticoids [5,18], Cyclosporine A, FK506 [19]	**–**	Arginine deficiency [57]
eNOS			
+	Shear stress [20] Exercise [21,22] Hypoxia (acute [23], chronic [24-26]) Hormones and autacoids: Estrogens (ERβ) [27,28], Insulin [29] Angiotensin II [2;30] (AT2 and calcineurin) [31] Drugs and toxins: Angiotensin converting enzyme inhibitors [32-34] Angiotensin II type 1 receptor antagonists [35-37] Some Ca^{++} channel blockers [38,39] β adrenoceptors antagonists [40] Statins [41,42] Nicotin [43] Nicorandil [44] Pertussis toxin [45]	+	Myristoylation[58] Palmitoylation[59] Serine 1177 phosphorylation: Stretching [60], AMPK [61], Insulin [62], Corticoids [63] Hsp90 [64], as in chronic hypoxia [65] Dynamin [66] Shear stress [67] Acute pacing [68] Hormones and autacoids: Bradykinin[69-71] Estradiol [72] VEGF [71] Acetylcholine [73] Substance P [74] Histamine [75] Angiotensin II [2] Drugs: β3-agonist [76,77] Ca^{++}channel blockers * [78] ACE inhibitors *[79] Statins † [80]
–	Lipopolysaccharides [46,47] LDL (native [48], glycosylated and oxidized [49]) Hyperglycemia [29] Cortisol [50] Milrinone [51] SNAP, 8-Br-cGMP and IBMX [52] Erythropoietin [53]	–	Caveolin-1 [81] Caveolin-3 [58] NOSTRIN [82] Phosphorylation changes: hyperglycemia (Ser1177–) [83] AMPK (Thr495+)[61] BH4 deficiency [84] ADMA [85] ROS [86]

* through BK potentiation; † through caveolin-1 reduction

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Online Table 2. Inotropic, lusitropic and chronotropic effects of NO in *unstressed* hearts, both at baseline and after β-adrenergic- and/or muscarinic-cholinergic stimulation

Model	Inotropic			Lusitropic		Chronotropic		
	Basal	**β**	**β+M2**	**Basal**	**β**	**Basal**	**β**	**M2**
eNOS-/-	= [1-12] ↑ [13]	↑ [1-3,12] = [4,5] not↓ [1]	= [3-4] not↓ [5]	= [1,2,6-8,12]	↑ [2] = [2]	↑ [1,12] = [2,4,8,13,14] ↓ [7,11,15-19]	= [4]	= [4] not↓ [14]
eNOS-TG	↓ [20,21]	= [20]	= [20]	= [20]		= [20,21]		
iNOS-/-	= [6, 22-29]	= [29]		= [6, 25-29]	= [29]	= [6, 22-26,28,29]	= [29]	
iNOS-TG	↑ [30] = [30,31]			= [30]		= [31] ↓ [31]		
nNOS-/-	↑ [32,33] = [1,15,34]	↓ [1] = [32] ↑ [32]		= [1] ↓ [32,33]	= [32]	↑ [35,36] = [1,34]		= [36] not↓ [36]
n+eNOS-/-	↑ [1] = [1]	= [1]		↓ [1]		↑ [1]		

β, beta-adrenergic; M2, Muscarinic type 2 ; -/-, knockout; TG, transgenic;
↑, enhanced effect in genetically modified mice compared with wild type/control (in absolute value); =, unchanged; ↓, decreased; not ↓, (contractility or heart rate) not decreased by β3agonist[1], carbachol[5,14] or vagal nerve stimulation[36] in knockout mice, while decreased in wild-type;

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Online Table 3. Myocardial NOS abundance and activity in the diseased heart

Disease	Abundance			Activity			
	nNOS	iNOS	eNOS	Total	nNOS	iNOS	eNOS
HCM	↑[1]	↑[2]	↓[3]	↓[1,3]		= [3]	
HF		↑[5-11] =[4, 12-15]	↓[6,8,16-18] = [9,12,15,19] ↑[12]	↑(early)[14, 21-23] ↓(late)[18,20]		↑[6,9,24,25]	↓[24] * = [9,15] * ↑[14, 25] *

HCM, hypertrophic cardiomyopathy; HF, heart failure; *, constitutive NOS (eNOS origin not proven)

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