**REVIEW**

# Cardiac electrophysiological effects of nitric oxide

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Nitric oxide (NO) synthetized by essentially all cardiac cell types plays a key role in the regulation of cardiac function. Recent evidence shows that NO modulates the activity of cardiac ion channels implicated in the genesis of the cardiac action potential and exerts anti-arrhythmic properties under some circumstances. We review the effects of NO on cardiac ion channels and the signalling pathways, including cGMP-dependent (protein kinase G and cGMP-regulated phosphodiesterases) and cGMP-independent mechanisms (S-nitrosylation and direct effects on G proteins) and finally the role of NO in the genesis of cardiac arrhythmias during ischemia−reperfusion, heart failure, long QT syndrome, atrial fibrillation, and sudden cardiac death.

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**Keywords**        Nitric oxide  •  Ion channels  •  Cyclic GMP  •  Cardiac arrhythmias

## 1. Introduction

Nitric oxide (NO) synthetized by essentially all cardiac cell types is an ubiquitous cellular messenger that plays a key role in regulating cardiac function.[1−5] NO is a highly diffusible gas that spreads very rapidly from its site of synthesis and a free radical highly reactive with other species, notably oxygen, superoxide and iron-containing haeme groups which act as NO scavengers. For this reason, the half-life of NO is limited to seconds and its effects are localized close to where it is synthetized. NO generated within the cardiomyocytes can exert intracrine effects or modify the functional properties of adjacent cardiomyocytes. NO generated from non-cardiomyocyte sources (coronary, endocardial, and endothelial cells, autonomic nerves and ganglia, and blood-formed elements) can exert direct effects on cardiomyocytes and indirect effects by modulating coronary blood flow and/or autonomic transmission.[1−5] The heart produces NO on a beat-to-beat basis in response to changes in coronary flow and myocardial loading. In rabbit hearts, NO levels reach peak values during diastole ($\sim$2.7 μM in the endocardium and $\sim$0.93 μM in the myocardium) and lowest during systole (0.67 and 0.26 μM, respectively); NO concentrations were 15% lower in rat hearts.[6]

The cardiovascular effects of NO have been analysed in excellent reviews.[1−5] However, its effects on cardiac ion channels and electrical activity are a forgotten aspect, despite its relevance. In this review, we analyse the effects of NO in the regulation of the cardiac ion channels implicated in the genesis of the cardiac action potentials (APs) (Figure 1) and its putative role in cardiac arrhythmias.

## 2. NO synthesis within the cardiomyocytes

NO biosynthesis from the substrate L-arginine is performed by NO synthase isoforms (NOSs): endothelial-eNOS/NOS3 and neuronal-nNOS/NOS1 are $Ca^{2+}$-calmodulin (CaM)-dependent, whereas inducible-iNOS/NOS2 produces much higher NO levels in a $Ca^{2+}$-independent manner. In cardiomyocytes, NOSs are expressed in distinct subcellular compartments and produce NO that then acts in highly site-specific targets to exert different, or even opposite effects, on cardiac function.[2,5,7] eNOS is mainly present in both coronary vascular endothelium and cardiac endocardium, to a lesser extent in cardiomyocytes and in some blood-formed elements (platelets and monocytes). In cardiomyocytes, eNOS is localized in the caveolae of the sarcolemma and T tubuli (associated with caveoline-3, b-adrenoceptors, muscarinic-M2 receptors, G proteins and voltage-gated ion channels), sarcoplasmic reticulum-SR (associated with ryanodine receptor $Ca^{2+}$ release channels, RyRC) and mitochondria.[2−4] Indeed, changes in the relative expression of ion channels, eNOS and signalling proteins in the caveolae could account for the described contradictory effects of NO on ion channels.[2−5,8] nNOS is expressed in cardiomyocytes, cardiac autonomic nerves, and intracardiac ganglia. In cardiomyocytes, nNOS is localized in the SR [associated with the $Ca^{2+}$-ATPase (SERCA2), RyRC], sarcolemma [colocalized with $Na^+$-$K^+$-ATPase and $Ca^{2+}$/CaM-dependent $Ca^{2+}$-ATPase (PMCA)] and mitochondria.[1−3,9,10] iNOS expression in cardiomyocytes, endothelial and vascular smooth muscle cells, fibroblasts, and inflammatory cells (macrophages) is stimulated by pro-inflammatory mediators (cytokines and endotoxin) and under pathological conditions (e.g. ischaemia−reperfusion injury, septicaemia, heart failure, some cardiomyopathies, etc.).[2−4]

## 3. NO signalling

NO exerts its biological effects through cGMP- and cGMP-independent signalling pathways. NO binds to the haeme moiety of soluble guanylyl cyclase (GC) to increase cGMP levels

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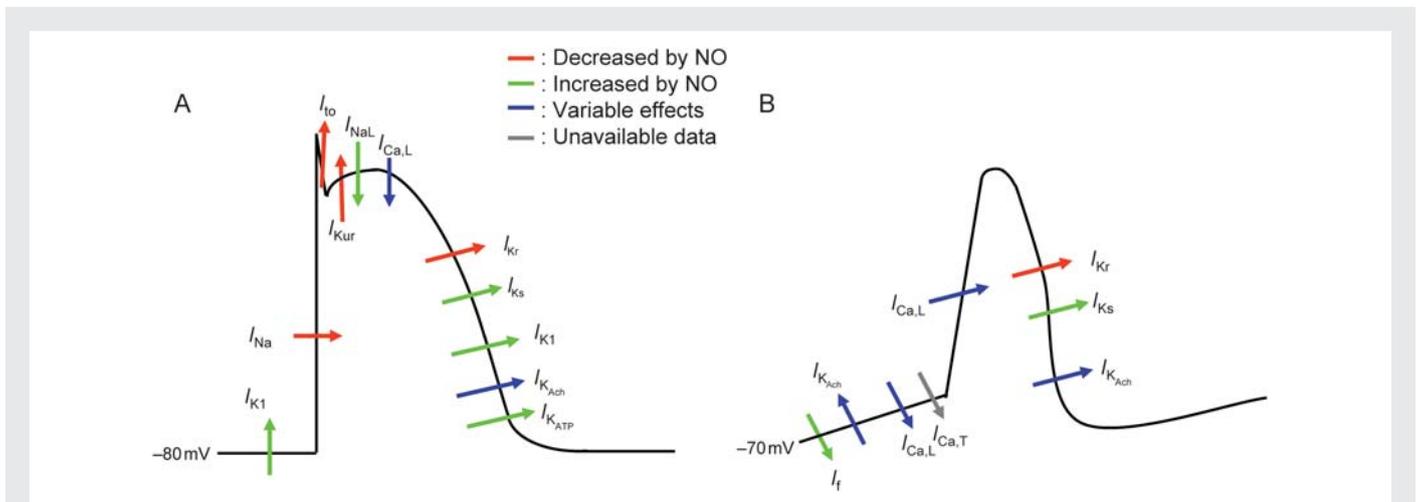
**Figure 1** Scheme of cardiac APs in (*A*) atrial and ventricular cells and (*B*) SAN cells showing the effects of NO on the currents participating in their genesis. Arrows indicate the ion movement direction, whereas colours indicate increase (green), decrease (red) or variable effects (blue) of NO.

which modulate the activity of cGMP-dependent protein kinase (PKG) and cGMP-regulated phosphodiesterases (PDEs).[11] cGMP-stimulated PDE2 enhances cAMP breakdown, whereas the cGMP-inhibited PDE3 increases intracellular cAMP levels. Species- and tissue-dependent expression of PDE subtypes partially account for the variable actions of NO−GC−cGMP pathway on cardiac channels.

NO also regulates the cardiac function through cGMP-independent pathways. S-nitrosylation, the covalent modification of a protein cysteine thiol by a NO group to generate an S-nitrosothiol, can occur upon formal oxidation of NO or by *trans*-nitrosylation in which the NO group is exchanged between donor and acceptor thiol.[12] S-nitrosylation is an ubiquitous post-translational modifying system through which NO modulates the activity of several cardiac channels.[12] *Table S1* provided as Supplementary material summarizes the effects of NO on cardiac ion channels, pumps, and transporters as well as the signalling pathways involved.

# 4. Effects of NO on cardiac ionic currents

## 4.1 Inward Na$^+$ current (I$_{Na}$)

In atrial and ventricular myocytes, the $I_{Na}$ generates the rapid depolarization upstroke of the AP and determines the cardiac excitability and the conduction velocity. Na$^+$ channels colocalize with caveolin-3 in cardiac sarcolemmal membrane.[8] The NO donor SNAP does not affect hH1 human Na$^+$ channels expressed in *Xenopus* oocytes or human embryonic kidney (HEK293) cells.[13] However, in native cardiomyocytes, NO may inhibit[14] or increase $I_{Na}$.[15]

NO inhibits $I_{Na}$ in isolated mouse and guinea pig ventricular myocytes without altering channel kinetics.[14] This inhibition is due to a decrease in open probability ($P_o$) without changes in single-channel conductance and involves the activation of both PKG and protein kinase A (PKA). However, in rat ventricular myocytes, NO donors induce a late Na$^+$ current ($I_{NaL}$) because Na$^+$ channels fail to inactivate completely or close and then reopen at depolarized potentials, i.e. during the plateau phase of the AP.[15] The $I_{NaL}$ could also be induced by endogenous NO generated following the activation of

NOSs activity with ionomycin. This cGMP-independent effect is blocked by *N*-ethylmaleimide (a thiol alkylating reagent preventing S-nitrosylation) and persists in cell-free outside-out patches, suggesting that it is produced by a direct *S*-nitrosylation of the α1-subunit (Na$_v$1.5) of the Na$^+$ channel.

## 4.2 L-type Ca$^{2+}$ current (I$_{Ca}$)

Cardiac depolarization opens L-type Ca$^{2+}$ channels (LTCC) generating an $I_{Ca}$ that is responsible for the AP plateau and triggers a larger release of Ca$^{2+}$ through the opening of RyRC. The $I_{Ca}$ is also responsible for phase 0 depolarization and the slow diastolic depolarization in sinoatrial (SAN) and atrioventricular nodal (AVN) cells.

NO produces contradictory effects on $I_{Ca}$, increasing,[16] decreasing,[13,17−24] or producing a biphasic effect.[25−28] In human atrial myocytes, the NO donor SIN-1 stimulates $I_{Ca}$, an effect that decreases at concentrations >1 μM.[26] The increase in $I_{Ca}$ is produced via cGMP-inhibited PDE3, which increases intracellular cAMP levels;[16,25,26] however, it has also been attributed to a cAMP-independent activation of PKG.[16] In ferret cardiomyocytes, an additional cGMP-independent direct activation of LTCC via S-nitrosylation/redox pathways has been postulated,[28] despite S-nitrosothiols inhibiting LTCC channels expressed in heterologous systems.[13] However, direct S-nitrosylation of cardiac LTCC was not confirmed in these studies. The inhibition of $I_{Ca}$ is related to activation of cGMP-stimulated PDE2[25,26] or a cGMP-PKG pathway not involving changes in cardiac cAMP levels.[16,23,27]

The α1C-subunit of the LTCC (Ca$_v$1.2) contains more than 10 cysteine residues that modulate channel gating and is constitutively S-nitrosylated in the mouse heart.[9,29] S-nitrosylation increases after isoproterenol exposure,[30] during ischaemia−reperfusion,[29,30] and in chronic atrial fibrillation (CAF).[31] Under these circumstances, NO-induced S-nitrosylation of LTCC reduces $I_{Ca}$,[13,29] SR Ca$^{2+}$ loading, and Ca$^{2+}$-induced cardiac injury, which represents an important cardioprotective mechanism (Section 5). nNOS-derived NO contributes to S-nitrosylation of the LTCC.[10,32] Indeed, nNOS deletion is associated with a higher LTCC activity,[32,33] whereas $I_{Ca}$ density decreases in nNOS overexpressing cells or after administration of SNAP to WT or nNOS$^{−/−}$ cardiomyocytes.[32,34,35] $I_{Ca}$

density is similar in WT and in $eNOS^{-/-}$ mice.[36] However, changes in $Ca_v1.2$ expression were not analysed.

### 4.2.1 Effects on $I_{Ca}$ stimulated by β-adrenergic stimulation

β-receptor stimulation activates LTCCs via adenylyl cyclase (AC)-cAMP-PKA-dependent phosphorylation of the channel. nNOS and eNOS differentially regulate the response to β-adrenoceptor stimulation: nNOS signalling potentiates, whereas eNOS depresses this response.[2,7,32,34,35,37] In ventricular cardiomyocytes, SAN and AVN cells NO inhibits $I_{Ca}$ stimulated by isoproterenol, cAMP, or for-skolin, an effect reversed by ODQ (a selective GC inhibitor) and PKG inhibitors.[18−20,23−25,27] The anti-adrenergic effect of NO involves the activation of cGMP-stimulated PDE2[19,20,25] and of PKG via a pertussis toxin-sensitive $G_i$ protein that inhibits the AC activity.[23]

### 4.2.2 Effects on muscarinic inhibition of $E_a$

M2-receptor activation stimulates eNOS, and NO and carbachol inhibit isoproterenol-stimulated $I_{Ca}$, producing an anti-adrenergic effect.[2] eNOS-released NO participates in the muscarinic inhibition because this response is absent in $eNOS^{-/-}$ ventricular myocytes, whereas the transfection of eNOS cDNA into cardiomyocytes of these mice restores the inhibition.[21] Others, however, found no changes in the chronotropic and inotropic responses to β-adrenergic and muscarinic agonists in cardiac preparations of $eNOS^{-/-}$ mice and that β-adrenergic stimulation and muscarinic inhibition of $I_{Ca}$ did not differ in cardiac myocytes from $eNOS^{-/-}$ and WT mice.[38,39] Thus, the role of eNOS-released NO in mediating muscarinic inhibition of isoproterenol-stimulated $I_{Ca}$ remains controversial.

## 4.3 Effects of NO on outward $K^+$ currents

Cardiac $K^+$ channels determine resting membrane potential (RMP), heart rate and shape, and AP duration (APD).[38]

### 4.3.1 Transient outward current ($I_{to}$)

In human atrial and ventricular myocytes, $I_{to}$, mainly carried by Kv4.3 channels, is responsible for early rapid repolarization (phase 1) and determines the height of the plateau, thus influencing the activation of other currents that control repolarization ($I_{Ca}$ and delayed rectifier $K^+$ currents). NO and NO donors inhibit currents generated by Kv4.3 and Kv4.2 ($I_{Kv4.3}$ and $I_{Kv4.2}$) channels in CHO cells and $I_{to}$ recorded in human atrial and mouse ventricular myocytes.[40] The effect of NO on $I_{Kv4.3}$ is independent of the cellular redox state or the activation of GC-cGMP-PKG pathway, protein kinase C (PKC), $Ca^{2+}$/CaM-dependent protein kinase II (CaMKII) and serine/threonine phosphatase 2B, but is suppressed by AC inhibitors. NO-induced activation of AC produces the subsequent simultaneous activation of cAMP-dependent serine−threonine phosphatase 2A (PP2A) and PKA. The activation of these signalling pathways was confirmed in human atrial myocytes, where simultaneous inhibition of PKA and PP2A completely prevents the effects of NO. $I_{to}$ density is similar in ventricular myocytes from WT and $eNOS^{-/-}$ mice and there are no differences in atrial APD or resting membrane potential between WT and $eNOS^{-/-}$.[40]

### 4.3.2 Ultra-rapid activating delayed rectifier current ($I_{Kur}$)

This current, carried by hKv1.5 channels, is recorded only in human atrial (but not in ventricular) myocytes. Thus, it plays a main role in atrial repolarization.[41] NO blocks hKv1.5 channels expressed in $Ltk^-$ cells without altering the time-dependent properties.[42] SNAP also inhibits the native current ($I_{Kur}$) recorded in mouse ventricular and human atrial myocytes. The effect of SNAP is partially reproduced by 8-bromo-cGMP and partially inhibited by selective inhibitors of GC and PKG and dithiothreitol (DTT), a thiol reducing agent. Furthermore, in SNAP-treated cells, biotin-switch assay demonstrates the presence of S-nitrosylated cysteines on hKv1.5 protein. Molecular modelling of Kv1.5 channel structure suggests that NO nitrosylates Cys331 and Cys346, located in the S2 segment of the channel protein, thus establishing hydrogen bonds with Ile262 (in S1 segment) and Arg342 (in segment S2), respectively. Therefore, NO inhibits hKv1.5 channels via activation of GC-cGMP-PKG pathway and S-nitrosylation of hKv1.5 protein.[42]

### 4.3.3 Rapidly activating delayed rectifier current ($I_{Kr}$)

This current, carried by Kv11.1 (human *ether-a-gogo-related-gene*, HERG) channels, is largely responsible for repolarization during phases 2 and 3 of the AP in most cardiac cells playing an important role in determining cardiac APD and refractoriness. NO donors and L-arginine inhibit, whereas L-NAME (a NOS inhibitor) increases hERG currents in *Xenopus* oocytes.[43] The effect of NO donors is prevented by NO scavengers and it is not mediated via the cGMP-PKG pathway. However, since L-arginine and NO donors counteract the stimulatory effect on hERG currents induced by the radical oxygen species-generating system $FeSO_4$/ascorbic acid (Fe/Asc), it was proposed that NO inhibits hERG currents via an interaction with radical oxygen species either generated under resting conditions or triggered by Fe/Asc, although S-nitrosylation was not investigated.

### 4.3.4 Slowly activating delayed rectifier current ($I_{Ks}$)

This current, which activates slowly during depolarization and deactivates slowly during repolarization, contributes to the late repolarization phase of the cardiac AP, being the main determinant of rate-dependent shortening of APD. As heart rate increases, Ks channels have less time to deactivate, leading to an accumulation of open channels and a faster rate of repolarization.[41]

NO donors enhance $I_{Ks}$.[44,45] This increase is barely affected by ODQ, whereas *N*-ethylmaleimide and DTT reverse this effect. Thus, the effects of NO are cGMP-independent, but related to S-nitrosylation of channel protein. In guinea pig ventricular myocytes, the $Ca^{2+}$ ionophore A23187 increases $[Ca^{2+}]_i$ and $I_{Ks}$ and shortens APD. The increase in $I_{Ks}$ is inhibited by L-NAC (a NO scavenger), W7 (a CaM inhibitor), and the eNOS inhibitor SMTU, but not by the nNOS inhibitor L-NIO.[45] Thus, CaM-dependent activation of eNOS confers the selective $Ca^{2+}$-sensitive modulation of $I_{Ks}$.

Ks channels are composed of the α-subunit Kv7.1 (encoded by KCNQ1), which presents two CaM-binding domains in the C-terminus and the β-subunit minK encoded by KCNE1.[41] SNAP induces S-nitrosylation of KCNQ1 at Cys445, a residue located in a consensus motif [(Lys,Arg,His)-Cys-(Asp,Glu)] between the CaM-binding domains and increases KCNQ1/KCNE1 current only in HEK293 cells cotransfected with CaM.[46] NO-mediated regulation of $I_{Ks}$ may play a role in APD shortening in response to increases in $[Ca^{2+}]_i$ or sympathetic stimulation and in the adaptation to heart rate changes.

### 4.3.5 Inward rectifier current ($I_{K1}$)

$I_{K1}$ is a strong rectifier that passes $K^+$ currents over a limited range of membrane potentials.[41] At potentials negative to $-40$ mV, $I_{K1}$ conductance is much larger than that of any other current, whereas upon

depolarization, inward rectifier channels close almost immediately, remain closed throughout the plateau, and open again at potentials negative to $-30$ mV. Thus, $I_{K1}$ plays a critical role in stabilizing the RMP and shaping the late phase 3 of the AP. In the human heart, inwardly rectifying channels Kir2.1, Kir2.2, and Kir2.3 contribute to $I_{K1}$, Kir2.1 channels being the major isoform underlying ventricular $I_{K1}$.

Physiological concentrations of NO increase $I_{K1}$ in human atrial myocytes and $I_{Kir2.1}$ in CHO cells transfected with Kir2.1 by augmenting channel $P_o$ and opening frequency without altering current amplitude.[47] NO facilitates the close-to-open transition and accelerates the channel closing kinetics. NO-induced increase in $I_{K1}$ remains in hypoxic conditions and in the presence of ODQ, but is prevented by DTT. Site-directed mutagenesis demonstrates that NO effects are mediated by selective S-nitrosylation of Kir2.1Cys76. In the primary sequence, Cys76 is surrounded by Asp71, Asp78, and Arg80 and is preceded by an aromatic amino acid (Phe73) that could act as catalysts for S-nitrosylation. Cys76 is highly conserved in the Kir2 family, which explains why SNAP also increases $I_{Kir2.2}$ and $I_{Kir2.3}$. The increase in the $I_{K1}$ is responsible for the hyperpolarization of the RMP and the marked shortening of the final phase of AP repolarization produced by NO in mouse[40] and human atria.[47]

Some cardiovascular diseases are accompanied by significant changes in myocardial NO production and/or bioavailability, decreasing in CAF or increasing during early ischaemia/reperfusion.[3] In CAF, oxidative stress markedly reduces atrial NO concentrations[48] and S-nitrosylated Kir2.1 proteins.[47] Conversely, CAF increases Kir2.1 expression level and $I_{K1}$ density.[49] Therefore, in CAF, the regulation of the expression level of Kir2.1 proteins and their post-translational modification by S-nitrosylation operate in opposite directions.

### 4.3.6 Acetylcholine-activated current ($I_{K_{ACh}}$)
In SAN, AVN, and atrial cells, M2-receptor stimulation activates $I_{KACh}$ (encoded by Kir3.1–3.4 channels) which hyperpolarizes the RMP, shortens the APD, and slows spontaneous firing rate of pacemaker SAN and AVN cells and AV conduction.[41]

NO donors do not modify $I_{K_{Ach}}$ in rabbit SAN[19,21] and AVN cells.[18−20] In rat atrial myocytes, they do not modify basal $I_{K_{Ach}}$ but potentiate the increase in $I_{K_{Ach}}$ elicited by acetylcholine, an effect not inhibited by ODQ.[34] However, NO does not modify human $K_{Ach}$ channels reconstituted in HEK293 cells in the absence or presence of acetylcholine.[24] These data suggest that NO produces the M2-dependent activation of a cGMP-independent $G_{i/o}$ protein, which ultimately opens $K_{Ach}$ channels.

### 4.3.7 ATP-sensitive $K^+$ current ($I_{K_{ATP}}$)
NO participates in cardiac ischaemic pre-conditioning and exerts cardioprotective effects on ischaemia−reperfusion.[2,3,46] These effects are suppressed by NOS inhibitors, reproduced with nitrates and absent in $eNOS^{−/−}$ mice.[2] Activation of $K_{ATP}$ (encoded by Kir6.1 and 6.2) channels in the sarcolemma or the mitochondria (mitoK$_{ATP}$) couples cell metabolism to membrane potential and exerts a cardioprotective effect during ischaemia which is abolished by mitoK$_{ATP}$ blockers (5-hydroxydecanoate and glibenclamide).[41,50]

In ventricular myocytes, NO donors do not increase $I_{KATP}$ but enhance the current induced by $K_{ATP}$ openers in whole-cell and cell-attached patches, but not in inside-out or outside-out patches, suggesting that NO-induced enhancement of $I_{KATP}$ needs the presence of intracellular mediators.[51,52] This potentiation is inhibited by Rp-CPT-cGMP (a PKG inhibitor) and protein PP2A and potentiated

by PKG activators (8-pCPT-cGMP), suggesting that NO−cGMP−PKG pathway contributes to phosphorylation opening of $K_{ATP}$ channels.

In rabbit ventricular myocytes and rat inner mitochondrial membranes reconstituted into lipid bilayers, SNAP directly activates mitoK$_{ATP}$ channels and potentiates the effects of the mitoKATP opener diazoxide.[53,54] These effects are blocked with selective mitoK$_{ATP}$ blockers and the NO scavenger carboxy PTIO. Moreover, in a rat model of anoxic pre-conditioning, the beneficial effects of SNAP are blocked by $K_{ATP}$ channel blockers, L-NAME and Rp-8-Br-PET-cGMP (a PKG inhibitor). Interestingly, eNOS expression is unaffected, whereas both iNOS expression and NO production are elevated by pre-conditioning.[55] These results confirm the involvement of NO−cGMP−PKG pathway in the cardioprotective function of mitochondrial $K_{ATP}$ channels and that iNOS, rather than eNOS, plays a central role against ischaemia-induced myocardial pre-conditioning.

### 4.3.8 Effects on two-pore domain potassium channels (K$_{2p}$)
Ischaemic pre-conditioning can be induced by metabolic inhibition with sodium cyanide (NaCN) in single cardiomyocytes perfused in glucose-free media. Under these conditions, an outward current is activated through TALK-1 (K$_{2P}$16.1) and TALK-2 (K$_{2P}$17.1) channels in guinea pig ventricular myocytes.[56] L-Arginine increases, whereas the NOS inhibitor L-NAME reduces the NaCN-induced current. Since K2P channels help to regulate cell volume,[41] it is possible that NO may play a role in the protection from prolonged ischaemia where cell swelling can induce apoptosis.

## 4.4 Effects on the pacemaker current ($I_f$)
The hyperpolarization-activated pacemaker current encoded by HCN1−4 channels is a mixed inward $Na^+/K^+$ current which activates slowly during phases 3 and 4 of the AP and inactivates slowly upon depolarization. $I_f$ underlies the slow diastolic depolarization in pacemaker SAN cells and its activation is modulated by cyclic nucleotides, being accelerated when intracellular cAMP levels increased.

In rabbit SAN cells, sodium nitroprusside (SNP) increases basal $I_f$, but decreases $I_f$ when pre-stimulated by isoproterenol, or cAMP, and both effects are abolished by methylene blue, a GC inhibitor.[57] However, SNP does not increase $I_f$ when it is pre-stimulated by non-specific PDE inhibitors. Thus, NO exerts a direct cGMP-dependent stimulatory effect on $I_f$ and an indirect inhibitory effect mediated by cGMP-stimulated PDE2 when cAMP production increases. NO-induced modulation of $I_f$ might play a role in the sinus tachycardia that accompanies pathological conditions associated with an increase in myocardial production of NO and sympathetic activation (e.g. heart failure).

## 4.5 Effects on RyRC
$Ca^{2+}$ release from the SR via the RyRC is essential in cardiac excitation−contraction coupling. The cardiac RyRC is a tetrameric complex containing $\sim$89 cysteines per monomer and S-nitrosylation of up to 12 thiol sites per RyRC progressively increases channel activation, an effect reversed by denitrosylation.[58] Indeed, S-nitrosothiols and NO donors increase $P_o$ in a single ventricular RyRC reconstituted in lipid bilayers through thiol oxidation, whereas sulphydryl reducing agents promote channel closure.[59] Conversely, others reported that L-arginine reduces the $P_o$, an effect prevented by NOS inhibitors and haemoglobin and reversed by thiol reducing agents.[60] These

contradictory effects may be related to the source (endogenously produced vs. exogenously administered) and concentration of NO.

In rat ventricular myocytes, the effects of NO on RyRC depend on the level of β-adrenergic stimulation and the state of PKA activation.[61] At low concentrations of isoproterenol, NO increases spontaneous $Ca^{2+}$ spark frequency (CaSpF, an index of RyRC activity), whereas at high concentrations (i.e. high PKA activation) NO decreases CaSpF. These changes are cGMP-independent but can be mediated via S-nitrosylation of RyRC and might have clinical implications in heart failure where β-adrenergic signalling is altered, RyRC are hyperphosphorylated, and NO production is increased.[2,3]

In cardiomyocytes, RyRC colocalize with nNOS.[7,10] $nNOS^{-/-}$, but not $eNOS^{-/-}$ mice, exhibit hypo-S-nitrosylation of RyRC, suggesting that nNOS is the isoform required for RyRC S-nitrosylation. However, eNOS-derived NO enhances RyRC $P_o$ and CaSpF in response to myocardial stretch through a cGMP-independent mechanism involving the activation of the phosphoinositide 3-kinase (PI3K)-Akt-eNOS pathway.[62]

## 4.6 $Na^+/Ca^{2+}$ exchange current ($I_{NCX}$)

Cardiac sarcolemmal $Na^+/Ca^{2+}$ exchanger (NCX) pumps $Ca^{2+}$ out of the cell electrogenically ($Na^+:Ca^{2+} = 3:1$) playing an important role in maintaining $Ca^{2+}$ homeostasis. However, during ischaemia–reperfusion, membrane depolarization and high $[Na^+]_i$ can cause $Ca^{2+}$ entry via reverse mode action of NCX leading to cardiac $Ca^{2+}$ overload. In human atrial myocytes, SNAP has no effect on the $I_{NCX}$.[47] Protein level and activity of NCX do not differ between $nNOS^{-/-}$ and WT myocytes.[33]

## 4.7 $Na^+–K^+$ pump current ($I_p$)

$Na^+–K^+$ ATPase and eNOS/nNOS colocalize with caveolin-3 in cardiac sarcolemmal membrane.[63] $Na^+–K^+$ ATPase transports three $Na^+$ out of the cell and two $K^+$ into cardiomyocytes regulating $[Na^+]_i$ and RMP. L-Arginine and SNP increase $I_p$, an effect abolished by GC and PKG inhibitors,[64,65] and cardiac sarcolemmal $Na^+–K^+$ ATPase activity decreases in $eNOS^{-/-}$ and/or $iNOS^{-/-}$ mice compared with WT controls.[66] However, the functional significance of these changes (i.e. the effect on RMP or AP characteristics) was not evaluated. Ischaemia inhibits $Na^+–K^+$ ATPase and contributes to intracellular $Ca^{2+}$ overload and contractile depression. In a rat model of global ischaemia, pre-treatment with a NO donor protects cardiac sarcolemmal NOSs and $Na^+–K^+$ ATPase against ischaemia-induced inactivation. All these results suggest that locally generated NO may serve to regulate cardiac $Na^+–K^+$ active transport.[63]

# 5. Effects of NO on cardiac arrhythmias

Coronary endothelial dysfunction and NOS inhibitors reduce the coronary effluent NO levels and increase the incidence and severity of ventricular arrhythmias in rat models of ischaemia–reperfusion,[67] whereas L-arginine[68] and NO donors, even at subvasodilatory doses, reduce ischaemia/reperfusion-induced ventricular fibrillation (VF) in rats and dogs.[2,69,70]

## 5.1 Role of eNOS

There is a great deal of evidence to support that eNOS-derived NO plays a cardioprotective role. $NOS3^{-/-}$ mice show no significant

differences in ECG intervals or intracardiac conduction parameters, but heart rate was slower than in WT mice.[71] However, digoxin produces a higher incidence of ventricular premature beats and ventricular tachycardia in $eNOS^{-/-}$ than in WT mice.[71] Ouabain also produces a higher rate of arrhythmic after-contractions in ventricular myocytes isolated from $eNOS^{-/-}$ mice than in WT myocytes, an effect inhibited by NO donors and ODQ.[72] Ouabain-induced after-contractions are related to the activation of a transient inward current ($I_{TI}$) generated by an oscillatory release of $Ca^{2+}$ from the SR when $[Ca^{2+}]_i$ is elevated. $eNOS^{-/-}$ myocytes develop an increase in ouabain-induced $I_{TI}$ compared with WT myocytes which is suppressed by NO.[72] Furthermore, $eNOS^{-/-}$ ventricular myocytes present longer APD values, higher $I_{Ca}$, and larger incidence of early/delayed after-depolarizations in response to isoproterenol compared with WT myocytes.[37] Similar results were observed with acute eNOS inhibition in WT myocytes. Thus, eNOS signalling exerts a cardioprotective role against β-adrenergic-mediated arrhythmias by inhibiting $I_{Ca}$, shortening $APD_{90}$, and decreasing $[Ca^{2+}]_i$.[37] Conversely, mice overexpressing eNOS present a lower incidence of spontaneous arrhythmic contractions and their hearts are less susceptible to ischaemia/reperfusion injury than WT mice.[2] Therefore, eNOS-derived NO may exert a cardioprotective effect against digitalis-, β-adrenergic-, and ischaemia/reperfusion-induced cardiac arrhythmias which could be of interest, particularly, under circumstances (i.e. heart failure) where eNOS expression decreases.[72]

## 5.2 Role of nNOS

The close association between nNOS and key $Ca^{2+}$ handling proteins (LTCC, RyRC, SERCA2) regulating $[Ca^{2+}]_i$ suggests that nNOS-derived NO may play a protective role against ischaemia/reperfusion- and $Ca^{2+}$-induced arrhythmias.[30,32,33] After myocardial infarction (MI), $nNOS^{-/-}$ ventricular myocytes present impaired S-nitrosylation of $Ca^{2+}$ handling proteins,[29,30,32] increased $I_{Ca}$, $Ca^{2+}$ transients and systolic and diastolic $Ca^{2+}$ levels, and enhanced the response to β-adrenergic stimulation compared with WT myocytes.[10,12,33,34] The increase in $I_{Ca}$ and in diastolic SR $Ca^{2+}$ release increases the electrical instability and the risk of ventricular arrhythmias and sudden cardiac death.[73] Indeed, after MI $nNOS^{-/-}$ mice present a higher incidence of ventricular arrhythmias and VF and a lower 30-day survival compared with WT mice[34] and the treatment with verapamil reduces the incidence of ventricular tachyarrhythmias, which confirmed a role for altered $Ca^{2+}$ handling in arrhythmogenesis. Similarly, nNOS inhibitors increase the amplitude of $I_{Ca}$ and the incidence of VF in models of ischaemia–reperfusion, effects that are reversed by NO donors.[74] Conversely, $Ca^{2+}$ transients, $I_{Ca}$, and diastolic $[Ca^{2+}]_i$ decreased in cardiomyocytes overexpressing nNOS[2,32] and after the administration of SNAP in $nNOS^{-/-}$ cardiomyocytes.[34] The reduction in $I_{Ca}$ density was attributed to the translocation of nNOS to the sarcolemma where it interacts with LTCC and inhibits the inward current. Furthermore, nNOS gene transfer decreases $I_{Ca}$ and normalizes the response to β-adrenergic stimulation in SAN cells by modulating cAMP-dependent modulation of $I_{Ca}$.[35] Thus, it can be proposed that nNOS-produced NO might exert a beneficial role in the regulation of $[Ca^{2+}]_i$ via S-nitrosylation of LTCC and SERCA2, which inhibits $I_{Ca}$, increases SR $Ca^{2+}$ uptake, and reduces diastolic $Ca^2$ leak and $[Ca^{2+}]_i$, particularly at high driving rates,[9] thus preventing the development of cardiac arrhythmias.[29,30,32] The cardioprotective effect of nNOS may be of particular interest in the presence of submaximal

β-adrenergic stimulation,[33] at faster driving frequencies,[10] and during ischaemia–reperfusion.[33,50]

# 6. Role of nNOS in sudden cardiac death

Dysregulated *S*-nitrosylation resulting from altered nNOS–target associations has been linked to the genesis of congenital long QT syndrome (cLQTS)[75] and sudden cardiac death.[76–78] In the sarcolemma, $Na_v1.5$ forms a complex with nNOS, PMCA4b (a negative regulator of nNOS) and $\alpha$1-syntrophin (SNTA1), a scaffold protein for nNOS and PMCA4b. A mutation in $\alpha$1-syntrophin (A390V) identified in a patient with a cLQTS selectively disrupts the nNOS-PMCA4b complex.[75] Release of PMCA4b increases the nNOS activity and released NO promotes the direct hyper-*S*-nitrosylation of $Na_v1.5$ and increases $I_{NaL}$ in HEK293 cells, which is the characteristic biophysical dysfunction for $Na^+$-channel-mediated cLQTS (LQT3). The $I_{NaL}$ increase is reversed by NOS inhibitors. These results suggest that SNTA1 is a cLQTS-susceptibility gene acting through $Na_v1.5$ (LQT12).

NOS1AP gene encodes CAPON (carboxy-terminal PDZ ligand of nNOS), a nNOS adaptor protein that regulates nNOS subcellular location. *NOS1AP* variants are associated with alterations in the QT interval in white adults.[76] Very recently, two single-nucleotide polymorphisms were independently associated with sudden cardiac death (rs10494366 with a 31% greater risk; rs12567209 with a 43% lower risk) in white (not in black) US adults.[77] In guinea pig ventricular myocytes, CAPON overexpression up-regulates nNOS (nor eNOS) and accelerates repolarization shortening the APD by inhibition of $I_{Ca}$ and activation of $I_{Kr}$, effects that are reversed by L-NAME.[78] These results provide an explanation for the association of CAPON gene variants with QT interval,[77,78] although it is unknown whether aberrant NOS activity or ion channel *S*-nitrosylation underlies these phenotypes.

# 7. Atrial fibrillation

Atrial fibrillation (AF), the most common cardiac arrhythmia, produces electrophysiological and structural changes (i.e. atrial remodelling) in the atria that promote its initiation and/or maintenance. Electrophysiological remodelling is characterized by a shortening of atrial APD and refractoriness.[49]

Several findings suggest that NO levels and bioavailabity are markedly reduced in the atria during CAF. In animal models, rapid atrial pacing decreases endocardial eNOS expression and NO bioavailability in the left atrium (not in the right atrium or aorta), probably due to a reduced eNOS activity under conditions of turbulent blood flow.[79] CAF also increases atrial NAD(P)H activity[48] and 3-peroxynitrite formation and decreases the plasma levels of nitrite/nitrate and platelet cGMP (two indicators of plasma NO levels).[80] Furthermore, NOSs contribute to atrial superoxide production,[48] suggesting that dysfunctional NOSs may play an important role in atrial oxidative injury and electrophysiological remodelling in CAF.[48] Consistent with this hypothesis, ascorbate, an antioxidant and peroxynitrite decomposition catalyst, attenuates atrial pacing-induced peroxynitrite formation and electrical remodelling and decreases the incidence of post-operative AF[81] and SNP markedly reduces the incidence (12 vs. 27%) and the duration of AF after coronary artery bypass grafting.[82]

NO modulates $Na^+$, $Ca^{2+}$, and $K^+$ ionic currents involved in atrial electrical remodelling. CAF-induced decrease in NO levels will increase outward $K^+$ currents through Kv1.5 and Kv4.3 channels.[40,42] These changes would shorten the plateau of the atrial AP, an effect that can contribute to the electrophysiological remodelling that maintains the arrhythmia. In fact, some $NOS3^{-/-}$ mice developed non-sustained AF.[71] However, in CAF LTCC, $\alpha$1C-subunit is hypernitrosylated, which decreases $I_{Ca}$ and exerts a cardioprotective effect by attenuating atrial $Ca^{2+}$ overload.[31] In certain circumstances, the maintenance of AF may depend upon the periodic activity of a small number of sustained, high-frequency, functional reentrant sources (rotors) localized mainly in the left atria and it has been found that specific blockade of $I_{Kur}$ and/or $I_{to}$ can terminate the rotor activity.[83] The underlying mechanism is related to a maximal prolongation of atrial APD at the plateau phase, rather than at the terminal phase of repolarization that leads to random tip meander and wavebreak, resulting in rotor termination. Indeed, at physiological concentrations, NO prolongs the plateau phase of the mouse atrial AP and lengthens the APD at the plateau phase of the human atrial CAF-modified AP as determined using a mathematical model.[40]

The role of NO gene polymorphisms on predisposition to AF remains controversial. An association of *eNOS G894T* (linked with a reduction in the eNOS promoter activity by ~50%) with AF has been described in patients with congestive heart failure,[84] but this finding was not confirmed.[85] However, the contemporary presence of *minK* 38G (reduces $I_{Ks}$) and *eNOS* −786C alleles (reduces NO production) synergistically increases the predisposition to non-valvular AF.[86] Unfortunately, no functional data are available in these studies.

# 8. Conclusions

There is evidence in animal models and humans that NO plays a physiological role in the regulation of almost all cardiac ionic channels and that cardiomyocyte nNOS/eNOS-released NO may represent a cardioprotective signalling pathway that lowers the threshold for arrhythmogenesis. Further studies are needed to confirm that agents that increase cardiac NO availability (i.e. nitrates, SNP, statins, ACE-inhibitors, angiotensin-receptor blockers) may exert both cardioprotective and anti-arrhythmic effects.

# Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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