Cardiac Mitochondrial Nitric Oxide: A Regulator of Heart Rate?

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Alterations in autonomic control and myocardial nitric-oxide (NO) production are likely linked to the development and progression of heart dysfunction. By focusing on heart rate, the complexity of the actions of NO at distinct levels throughout the autonomic nervous system and its relationship with other regulators can be demonstrated. Given the multiple and opposing actions of NO on cardiac control, it is difficult to interpret a response after a global intervention in the NO system. The diversity of intracellular pathways activated by NO, and their differing sensitivities to different levels of NO, might account for some aspects of reported specific but opposite effects. We discuss factors that might contribute to this diversity of actions. A proper elucidation of the effects of NO on metabolic pathways and on energy generation could lead to novel therapeutic strategies aimed at the early treatment of heart dysfunction.

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Nitric oxide (NO) is involved in the control of cardiovascular function in physiological conditions and in heart diseases. As the prototypical endothelium-derived relaxing factor, NO is a primary determinant of blood vessel tone and thrombogenicity. In the context of heart tissue, these functions themselves are sufficient to justify the growing interest in NO as a regulator of cardiac function. However, despite the extensive literature, the exact sites and nature of NO action in the heart are as yet uncertain, while the earlier hypothesis that all three genomic forms of NO synthase (NOS) are active in the heart, have given rise to several intriguing questions. The classic concepts linking heart function with NO are: (i) NO is a regulator of cardiac function through direct action on the myocardium, and also by indirect vascular-dependent mechanisms[1] and (ii) the three known genomic isoforms of NOS[2] are present and functionally active in the heart. Gonzales *et al.*[3] and Zaobornyj *et al.*[4] challenged the second concept and reported that NO is produced in physiological conditions in the myocardium in relevant quantities by two of the isoforms of NOS: (i) an isoenzyme located in the mitochondria, known as mitochondrial NOS (mtNOS) and (ii) an isoenzyme located in the cytosolic fraction, the endothelial NOS (eNOS). The mechanisms by which NO regulates heart contractility and contraction rate, and the relation between the heart cycle and diffusion of NO between mitochondria and cytosol are physiological processes that are only now beginning to be understood. In this paper we attempt to revisit the major concepts about the influence of NO on heart rate, with special focus on the role of mitochondrial NO.

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NO AND HEART RATE MODULATION

During the past decade, it has been found that NO plays a major role in physiological and pathophysiological processes that control many aspects of myocardial function.[5] However, establishing the exact role of NO in these processes has proven to be difficult, especially from the multicellular and whole-heart points of view. This is not surprising, given the site-specific sources of NO in the heart and the varied concentration-dependent effects that it produces. In the light of the literature on this topic, there are some parameters that may be relevant when evaluating the cardiac activity of NO. These are: the concentration of NO released or added, the intracellular location of the NOS isoforms, the prior adrenergic or cholinergic stimulation, the cellular redox state, the ambient temperature, the presence of immune response, the type of cell (atrial or ventricular), the chemical species involved, and the specific physiological function that is being studied. Different experimental protocols proved helpful in looking for promising approaches to the interpretation of the action of NO on the pacemaker function. As regards the chronotropic response, early evidence appeared to support a role for cardiac NO in the autocrine regulation of cholinergic signaling. An early study showed that cholinergic inhibition of cardiac chronotropy could be blocked by endogenous NOS inhibition or by NO scavengers in cultured, spontaneously beating, neonatal rat ventricular myocytes, and in atrio-ventricular node cells.[6] This concept was developed further by Han and co-workers who demonstrated that the use of NOS inhibitors, or eNOS gene knockout mice prevented cholinergic inhibition of the calcium channel in adrenergically prestimulated rabbit sinoatrial node cells.[7] Additionally, in myocytes from neonatal $eNOS^{-/-}$ mice, Feron *et al.* showed that the negative chronotropic effect of carbamylcholine (a stable analogue of acetylcholine) on the spontaneous beating rate was restored to normalcy by transfecting

with wild-type eNOS.[8] However, caution should be exercised while attempting to extrapolate experimental results from studies on cells of neonatal mice to applications involving autonomic control of heart rate in humans. Rather than conducting *in vitro* experiments with myocytes, it would probably be more useful to work with isolated sinoatrial preparations, or carry out *in vivo* studies, given the complexity of the neural, autocrine, and paracrine regulatory functions. NO donors increase the heart-rate response to direct vagal nerve stimulation both *in vitro* and *in vivo*, through a cGMP-dependent pathway,[9] enhancing the release of acetylcholine from parasympathetic nerve terminals. As expected, atria-vagus preparations isolated from nNOS knockout mice show a higher basal heart rate[10] and an impaired vagal bradycardia compared with the wild-type control.[11] On the other hand, a potential role for NOS in the sympathetic autonomic control of heart rate, through β-receptors, has been widely suggested.[12] Many reports have shown that inhibition of NOS induces an increase in the chronotropic response to β-agonists at the cellular, myocardial tissue, and whole-animal levels.[13,14] Experiments using eNOS$^{-/-}$ mice *in vivo*[15] or Langendorff-perfused hearts demonstrated augmented responses to β-agonists.[16] By contrast, β-adrenergic chronotropic response was found to be significantly attenuated in the experiments where the targeted expression of eNOS in cardiomyocytes was amplified by transgenic overexpression[17] or by other means,[1] thereby suggesting that the effect of eNOS on cardiac conduction and excitability is antagonized by the β-adrenergic system. Some of the discrepancies in these studies could have been caused by methodological differences, differences in the age of the knockout animals used, development of hypertension secondary to a high total peripheral resistance, the ambient temperature during the experiments, the level of prior adrenergic stimulation, and the use of whole cells rather than the permeabilized patch clamp technique. In addition, several studies show that myocardial chronotropy is affected by NO in a biphasic manner. Whilst low doses of sodium nitroprusside increase heart rate, higher doses have a negative chronotropic effect.[18] We recently reported that acute NOS inhibition with N^{G}-nitro-L-arginine methyl ester (L-NAME) induces an increase in blood pressure accompanied by tachycardia in complete-autonomic-blocked anesthetized rats (**Figure 1a,b**). In normal rats, the same doses of L-NAME increased arterial pressure but did not modify the heart rate. This may be because of the automatic regulation of heart rate in normal rats by suitable adjustment of the vagal and adrenergic tones. We demonstrated that the effect of L-NAME on blood pressure is independent of sympathetic and parasympathetic influences and is probably the result of a direct vasoconstrictor effect caused by the decrease in vascular NO synthesis. This result agrees with published data, and demonstrates that the NO-dependent

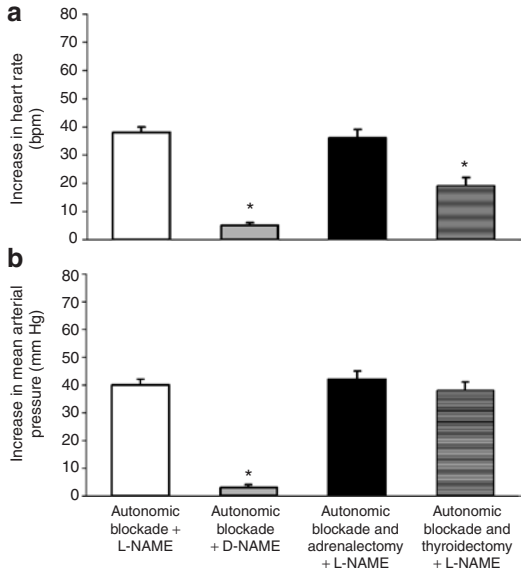

Figure 1 | Effect of L-NAME (7.5 mg/kg) on the heart rate and mean arterial pressure in autonomic-blocked rats. (**a**) heart rate; (**b**) mean arterial pressure. Experiments on autonomic-blocked control rats were performed using D-NAME. Data are presented as mean values ± s.e.m., $n = 18$ rats/group, $^{*}P < 0.05$, significantly different from autonomic-blocked rats treated with L-NAME. Reprinted from ref. 35.

systemic vasodilatation occurs in the absence of a functional autonomic nervous system.[19,20] We observed that the tachycardic response induced by L-NAME reached the maximum value at 30 min after administration. This would suggest that, the activation of humoral mechanisms, physical factors such as shear stress, and the direct effect of L-NAME may all be involved in this chronotropic response.[21] Experiments carried out in control animals, namely autonomic-blocked rats, using N^{G}-nitro-D-arginine methyl ester (D-NAME), showed no effect on arterial pressure and heart rate when compared against basal values (**Figure 1a,b**). We thereby confirmed the specificity of L-NAME-induced pressor and chronotropic responses. On the basis of our findings from *in vivo* experiments, we evaluated the effect of L-NAME on pacemaker and contractile activities in right atria isolated from autonomic-blocked rats. We showed that, under our experimental conditions, the addition of L-NAME at doses equivalent to those used *in vivo* experiments did not affect either chronotropic or inotropic activities. Because other authors have shown that the NO system is related to a bradycardic effect (experiments in NOS knockout mice, applications of NO donors and/or NOS inhibitors),[11,22], our findings can be interpreted, in this context, as suggesting a direct negative chronotropic effect of NO on the pacemaker activity. Our results support the idea that the L-NAME-induced tachycardia observed in *in vivo* experiments is not caused by direct action of the inhibitor on the

pacemaker activity. Working with right atria isolated from rats, Riado *et al.* showed that L-NAME did not affect the beat rate,[23] whereas others have reported that low doses of an NO donor or 8-bromo-cGMP increase the spontaneous beating rate in atria isolated from guinea-pig and in sinoatrial node cells from rabbit, by activation of the hyperpolarization inward current.[24] Taking our results into consideration, it is probable that other humoral factors may be involved in the L-NAME-induced tachycardia in autonomic-blocked rats.[25]

NO AND OTHER MODULATORS OF HEART RATE

Catecholamines, secreted by adrenal glands, produce positive inotropy and chronotropy mediated by β-adrenoceptors. It has been reported that the NOS inhibitor L-NAME inhibits acetylcholine-induced catecholamine secretion in bovine chromaffin cells,[26] and that the NO donor enhances nicotine-induced catecholamine secretion in bovine chromaffin cells in culture.[27] However, Torres *et al.* have reported contradictory results,[28,29] reporting that interaction between NO, catecholamines, and heart rate in the denervated heart are not observed. Therefore, in our laboratory, we recently studied this inter-relationship in autonomic-blocked animals. We observed that bilateral adrenalectomy did not modify the L-NAME-induced tachycardia or the pressor response in autonomic-blocked rats (**Figure 1a,b**). This finding suggests that catecholamines are not involved in the chronotropic response induced by NO inhibition in the denervated heart.[25]

On the other hand, the thyroid gland has at least some effect on every organ in the body including the heart. In hypothyroidism the heart muscle is weakened in both its contraction phase and its relaxation phase. This means that the heart cannot pump as vigorously as it should, and the amount of blood ejected with each heart beat is reduced. Furthermore, hypothyroidism reduces the amount of endothelial NO, thereby causing blood vessels to stiffen. These findings confirm the existence of a functional relationship between thyrocytes and endothelial cells, and therefore with the NO pathway. We recently showed, in *in vivo* experiments, that the pressor effect induced by L-NAME in autonomic-blocked rats is independent of the action of the adrenal and thyroid glands[30] (**Figure 1b**). These data are also in agreement with those from others reports that have shown that oral administration of methimazole maintained normal blood pressure levels in L-NAME-treated rats at 25 days after induction of hypertension.[31] In respect of heart rate, our group observed tachycardia following L-NAME administration in autonomic-blocked rats. This phenomenon was not modified by adrenalectomy, but was counteracted to an extent of ~50% by thyroidectomy (**Figure 1a**). These observations suggest that thyroid hormones play a protective homeostatic role against the chronotropic effect of NO. Furthermore, autonomic-blocked rats with a functional NO system showed an increase in total NOS activity in the atria but not in the ventricles at 150 min after thyroidectomy.[30] This finding allows us to hypothesize that the decrease in the plasmatic T_3 levels may be one of the stimuli for the increase in atrial NOS activity, and that this constitutes evidence to show that thyroid hormones act as a buffer to moderate the cardiovascular effects of NO. These observations have potential clinical relevance, because an excess of these hormones in conjunction with reduced NO production in diseases such as atherosclerosis, diabetes, and essential hypertension, may aggravate their respective pathogenic effects. Moreover, NO deficiency may represent a link between metabolic and cardiovascular disease. Further study and investigation of the interaction between NO and the thyroid hormones at the cellular level in the main target organs, i.e., heart, blood vessels, and kidney, will open new perspectives in relation to the treatment of cardiovascular diseases.

HEART MITOCHONDRIAL NO

Mitochondrial dysfunction is present in many pathological situations that lead to cardiovascular diseases. There is mounting evidence pointing to NO as a pivotal molecule in mitochondrial physiology. At nanomolar concentrations, NO binds to cytochrome oxidase (complex IV) in competition with O_2, inhibiting the activity of the oxidase reversibly.[32] At slightly higher concentrations, NO inhibits electron transfer at ubiquinol-cytochrome c reductase (complex III), while at even higher concentrations (by the formation of peroxynitrite), it inhibits NADH-ubiquinone-reductase activity (complex I).[33,34] In our laboratory, we have shown that autonomic blockade causes a rapid and significant (61%) decrease in whole-heart NO production, with decreases of 74 and 52% in mtNOS and eNOS activity, respectively. The downregulation of eNOS expression could be a consequence of the absence of vagal regulation on the enzyme expression. Moreover, we showed that the decreased activity of mtNOS in autonomic-blocked rats (74%) and in autonomic-blocked rats with thyroidectomy (40%) was correlated with the reduction in (i) mtNOS functional activity (55–60 and 16–21%, respectively), (ii) western blot protein expression (70 and 53%, respectively), and (iii) right atrial NADPH-diaphorase histochemical activity (55 and 21%, respectively) (**Figure 2**).[35] This NOS isoform reacted with antibodies against iNOS in western blot analysis, and similar observations were made in mitochondria isolated from other organs, such as liver[36] and kidney[37] where also iNOS-like immunoreactivity was found. Despite the fact that cloning has demonstrated mtNOS to be a genomic transcript of nNOS,[38] the apparent discrepancies relating to mtNOS reactivities with the available anti-NOS antibodies can be considered as arising from crossreactions and homology in NOS sequences.[39] Immunohistochemical analysis using anti-iNOS antibodies, an antibody with conjugated gold particles, and electron microscopy confirmed the localization of the mtNOS

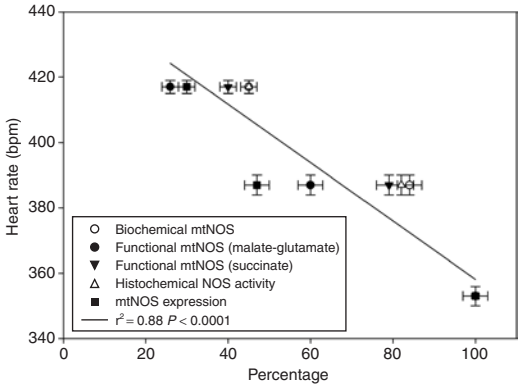

Figure 2 | Statistical correlation involving heart rate and mtNOS activity as in biochemical activity, functional activity, histochemical activity and protein expression. Points correspond to mean values relating to nine animals from each group. $r^2 = 0.88$; $P < 0.001$.

in the inner mitochondrial membranes.[35] Our results therefore support the concept that mtNOS is one of the main NO sources in the heart in our experimental condition. However, we cannot rule out the possibility of there being more than one mtNOS variant, depending on the type of tissue and animal species examined. On the other hand, we showed that thyroidectomy partially prevented the marked decrease in mtNOS expression and biochemical mtNOS activity and also the increase in heart rate produced by L-NAME in autonomic-blocked rats. The concept of hormonal regulation of mtNOS activity was advanced by Carreras *et al.*,[36] who observed that mtNOS activity and protein expression in liver and skeletal muscle are regulated by the thyroid status. Thyroidectomy-induced upregulation of mtNOS protein expression and activity in the autonomic-blocked animals is in agreement with previous reports in which hypothyroidism was associated with upregulation of mtNOS activity and administration of T_3 was associated with downregulation of the enzyme.[36] In addition, our findings show that mitochondria exhibited an increase in succinate-supported respiration after autonomic blockade, thereby indicating a close and rapid regulation by the autonomic nervous system of the level of mitochondrial proteins in the heart. The downregulation of NOS activity after autonomic blockade should lead to decreased levels of cGMP and increased rates of O_2 uptake and adenosine triphosphate (ATP) synthesis in the cardiomyocytes, considering the effects of NO on guanylate cyclase and cytochrome oxidase.[39] Lower levels of cGMP and a higher ATP availability should both stimulate the molecular mechanism to a higher rate of impulse generation by the sinusal node. A possible hypothesis based on the mitochondrial NO steady-state levels in the sinusal node would focus on the NO-mediated regulation of O_2 uptake and ATP levels, and would perhaps suggest that decreased and suboptimal rate-limiting levels of ATP lead to decreased rates of sinusal activity and heart contraction.[35]

In summary, the complexity of the actions of NO at distinct levels of the autonomic nervous system and the cardiovascular system make it difficult to interpret responses following a global intervention in the NO system. For the moment, the relationship between NO and the mitochondria, against the background of the activities of other modulators of heart rate regulation, provides fertile ground for new research which could lead to novel therapeutic strategies aimed at the prevention or early treatment of heart dysfunction.

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