Long-Term Nitric Oxide Inhibition and Chronotropic Responses in Rat Isolated Right Atria

Sonia R. Riado, Angelina Zanesco, Louis Allen Barker, Iara M.S. De Luca, Edson Antunes, Gilberto De Nucci

Abstract—The long-term administration of nitric oxide synthesis inhibitors induces arterial hypertension accompanied by left ventricular hypertrophy and myocardial ischemic lesions. Because the enhancement of sympathetic drive has been implicated in these phenomena, the current study was performed to determine the potency of β-adrenoceptor agonists and muscarinic agonists on the spontaneous rate of isolated right atria from rats given long-term treatment with the nitric oxide inhibitor \(N^\text{m}\)-nitro-L-arginine methyl ester (L-NAME). Atrial lesions induced by long-term treatment with L-NAME were also evaluated. Long-term L-NAME treatment caused a time-dependent, significant (\(P<0.05\)) increase in tail-cuff pressure compared with control animals. Our results showed that the potency of isoproterenol, norepinephrine, carbachol, and pilocarpine in isolated right atria from rats given long-term treatment with L-NAME for 7, 15, 30, and 60 days was not affected as compared with control animals. Addition of L-NAME in vitro (100 \(\mu\text{mol/L}\)) affected neither basal rate nor chronotropic response for isoproterenol and norepinephrine in rat heart. Stereological analysis of the right atria at 15 and 30 days revealed a significant increase on amount of fibrous tissues in L-NAME–treated groups (27±6.2% and 28±1.3% for 15 and 30 days, respectively; \(P<0.05\)) as compared with the control group (22±1.1%). Our results indicate that nitric oxide does not interfere with β-adrenoceptor–mediated and muscarinic receptor–mediated chronotropic responses. (Hypertension. 1999;34[part 2]:802-807.)

Key Words: adrenergic receptor agonists ■ receptors, muscarinic ■ receptors, adrenergic, beta ■ blood pressure ■ nitric oxide

The primary autonomic regulation of sinoatrial nodal function is by actions of sympathetic and parasympathetic systems. The effects of parasympathetic stimulation on the rat heart are mediated by muscarinic M2-receptors, whereas the sympathetic actions are mediated by β-adrenoceptors. \(\beta_1\), \(\beta_2\), and \(\beta_3\)-adrenoceptors are known to coexist in atria from several animal species, including humans. In rats, under physiological conditions, only \(\beta_1\)-adrenoceptors mediate chronotropic response.

A role for nitric oxide (NO) has been proposed in the modulation of sympathetic and parasympathetic neurotransmission in different tissues. Regarding the effects of NO on the chronotropic responses in the heart, little is known. Long-term administration of NO synthesis inhibitors induces arterial hypertension accompanied by left ventricular hypertrophy and myocardial ischemic lesions. The increase in the activity of the renin-angiotensin system and enhancement of sympathetic drive have been implicated in these phenomena. However, the underlying mechanisms by which long-term NO blockade induces arterial hypertension and other cardiovascular changes remain unclear. Therefore, the current study was performed to determine the potency of β-adrenoceptor agonists and muscarinic-agonists on the spontaneous rate of isolated right atria from rats given long-term treatment with the NO inhibitor \(N^\text{m}\)-nitro-L-arginine methyl ester (L-NAME). We have also evaluated atria lesions induced by long-term treatment with L-NAME.

Methods

Experimental Design

Experiments were performed in 164 male Wistar rats (150 to 200 g) that were provided by animal care of Paulista State University (UNESP, Botucatu). They were randomly divided into 2 experimental groups: control rats that received tap water alone and treated rats that received L-NAME (20 mg/rat per day, dissolved in the drinking water). The experiment lasted up to 8 weeks. All procedures were designed in accordance with the animal care guidelines of the State University of Campinas.
Blood Pressure Measurement
The mean arterial blood pressure was measured by use of a modified tail-cuff method in awake animals. The measurements were performed weekly and 24 hours before the animals were killed.

Functional Assays With Isolated Right Atria
At 7, 15, 30, or 60 days after L-NAME treatment, the animals were anesthetized with halothane and euthanized by stunning and exsanguination. The hearts were rapidly removed. The right atria were isolated and mounted in a water-jacketed tissue chamber (20 mL volume) containing Krebs-Henseleit buffer, pH 7.3 to 7.5, at 37°C and gassed with 95% O2-5% CO2. The composition of the Krebs-Henseleit buffer was (mmol) NaCl 124; KCl 4.75; MgCl2 1.30; CaCl2 2.25; NaHCO3 25.0; NaH2PO4 0.6; dextrose 10.0; sodium ascorbate 0.3; and disodium EDTA 0.03. Ascorbate and EDTA (Sigma Chemical Co) were added to inhibit the oxidation of catecholamines. One hour was allowed to obtain a stable basal rate. Another set of experiments in right atria from naive rats was performed by the addition of L-NAME (100 μmol/L) in the tissue bath.

Construction of Concentration-Response Curves
Concentration-response curves for the positive chronotropic actions of isoproterenol and norepinephrine and negative chronotropic actions of carbachol and pilocarpine (Sigma Chemical Co) were constructed by the cumulative variation of agonist concentration at one-half log unit increments.

All concentration-response data were evaluated for a fit to a logistics function in the form

\[ E = E_{\text{max}} \left( \frac{1}{1 + \left( \frac{c}{x} \right)^n} \right) + \Phi \]

where \( E \) is the increase in rate above basal; \( E_{\text{max}} \) is the maximum response that the agonist can produce; \( c \) is the logarithm of the EC50, the concentration of agonist that produces half-maximal response; \( x \) is the logarithm of the concentration of agonist; the exponential term \( n \) is a curve-fitting parameter that defines the slope of the concentration-response line; and \( \Phi \) is the response observed in the absence of added agonist. Nonlinear regression analyses to determine the parameters \( E_{\text{max}} \), log EC50, and \( n \) were done with the use of GraphPad Prism (GraphPad Software) with the constraint that \( \Phi = 0 \).

Stereological Procedures
Stereological analyses were performed according to the method described by Aherne. The atria were dissected and fixed in formalin for 24 hours. The right atria were then embedded in paraffin, and 5-μm sections were stained with Masson’s trichrome. Analysis of the slides was performed blinded on a light microscope (Zeiss), and the relative volume occupied by each element of the right atrium (myocardial fibers, fibrous tissue, or vessels) was measured with a special ocular containing a 25-point reticulum (5 parallel lines with 5 points each, kpl 8X, Zeiss, Germany). For counting, 50 microscopic fields were evaluated and the relative volume (Ppi) occupied by each component was calculated as follows: \( \text{Ppi} = \frac{p}{P} \), where \( p \) is the number of reticular points hitting each cardiac element and \( P \) is the total number of reticular points. We assumed fibrous tissue as the sum of postnecrotic fibrous scars and interstitial and perivascular fibrosis in treated animals. In the control animals, fibrous tissue means the normal connective tissue present in right atria.

Statistical Analysis
All values are expressed as mean±SEM. The program InStat (GraphPad Software) was used for statistical analyses. Where appropriate, 1-way ANOVA followed by a Bonferroni multiple comparisons post hoc test were performed to determine if the treatments had an effect. In some cases, a paired or unpaired Student’s t test was used. A level of \( P<0.05 \) was accepted as significant.

Results
Time Course of L-NAME Treatment on Blood Pressure
Long-term L-NAME treatment caused a time-dependent, significant (\( P<0.05 \)) increase in tail-cuff pressure, as evaluated at 7, 15, 30, and 60 days (132±5, 143±2, 150±3, and 165±9 mm Hg, respectively) compared with control animals (128±5 mm Hg at 60 days).

Figure 1. Concentration-response curves to isoproterenol in isolated right atria from L-NAME–treated rats at 7, 15, 30, and 60 days. ○, Control animals; ●, treated animals. Data are mean±SEM for 5 to 12 experiments.
Potency and Efficacy of β-Adrenergic and Muscarinic Agonists

**L-NAME Treatment**

There were no alterations on the potency of isoproterenol (Figure 1) and norepinephrine (Figure 2) at 7, 15, 30, and 60 days after L-NAME treatment. Although there was a tendency of rightward shift on the concentration-response curves to isoproterenol at 15, 30, and 60 days after L-NAME treatment, the pEC\(_{50}\) values were not statistically significant (Table 1). Negative chronotropic responses to muscarinic agonists carbachol and pilocarpine were also unaffected by L-NAME treatment at all times studied (Figures 3 and 4). L-NAME treatment had no effect on the spontaneous rate of isolated right atria. Maximal responses produced by each used agonist were also not affected by L-NAME treatment (Table 2).

**In Vitro Studies**

Addition of L-NAME (100 μmolar/L) in vitro in rat isolated right atria had no effect on the basal rate (250±10 bpm) compared with control animals (245±7 bpm). The pEC\(_{50}\) for isoproterenol and norepinephrine were also unchanged by L-NAME (8.66±0.07 and 7.92±0.07, respectively) in comparison to control animals (8.79±0.04 and 7.84±0.03, respectively).

**Stereological Studies**

Stereological analysis of the right atria at 15 and 30 days revealed significant (P<0.05) atrial lesions as assessed by the increase on amount of fibrous tissues in L-NAME–treated groups (27±2.3% and 28±1.3%, respectively) compared with the control group (22±1.1%).

**Discussion**

Our study shows that although long-term treatment with L-NAME induced atrial lesions, there were no repercussions in the responses to both sympathetic and parasympathetic agonists, indicating that NO is not involved in either receptor regulation and/or transduction mechanisms. This is further supported by the finding that addition of L-NAME in vitro had no effect on basal rate and chronotropic response for isoproterenol and norepinephrine.

NO is believed to play an important role in the regulation of sympathetic activity in the brain stem, including the ventrolateral medulla and nucleus tractus solitarius. Additionally, hypertension induced by long-term L-NAME treatment produces increased plasma levels of epinephrine (but not norepinephrine) through activation of the adrenal-medullary system. It is well established that overstimu-
lation of adrenoceptors may induce their desensitization, leading to a decrease in receptor density and/or reduction of agonist efficacy. The high epinephrine plasma levels found in this experimental model of hypertension would therefore be expected to alter the adrenoceptor-mediated chronotropic responses. However, in the current study, we found that hypertension induced by long-term L-NAME administration had no effect on the

Figure 3. Concentration-response curves to carbachol in isolated right atria from L-NAME–treated rats at 7, 15, 30, and 60 days. ○, Control animals; ●, treated animals. Data are mean±SEM for 5 to 12 experiments.

Figure 4. Concentration-response curves to pilocarpine in isolated right atria from L-NAME–treated rats at 7, 15, 30, and 60 days. ○, Control animals; ●, treated animals. Data are mean±SEM for 5 to 12 experiments.
sensitivity of right atria for positive chronotropic responses to norepinephrine and isoproterenol. Thus, similar pEC_{50} values for positive chronotropic response to selective β_{1}-agonist norepinephrine were seen for both control and treated groups. The concentration-response curves to the nonselective β-agonist isoproterenol showed a slight rightward shift at 15, 30, and 60 days after L-NAME treatment (approximately 2-fold), but this was not statistically different when a multiple comparison test was used. Similarly, we verified that addition of L-NAME to the organ bath failed to alter basal rate and chronotropic response mediated by β-adrenoceptors in right atria from naïve rats. These results suggest that neither long-term nor short-term blockade of NO synthesis affects the heart β-adrenoceptor–mediated responses. Our results corroborate previous studies showing that the substrate L-arginine and/or the NO donors influence neither chronotropic nor inotropic responses in rat right atria.

The influence of NO on the parasympathetic nervous system in the heart is a controversial matter. L-NAME in vitro acts as a muscarinic antagonist blocking M_{2}- and M_{1}-receptors in isolated tissues, displacing the concentration-response curves. In mice lacking endothelial NO synthase, the myocyte responsiveness failed to be stimulated by carbachol, suggesting a role for NO in coupling muscarinic receptor activation in cardiac myocytes. However, our current results show that the negative chronotropic responses to the full agonist, carbachol, were not changed in animals given long-term treatment with L-NAME. Similarly, this treatment had no effect on the potency of the partial agonist pilocarpine, whose actions are more sensitive to detect changes in receptor number and/or coupling mechanisms. These findings show that NO does not interfere with muscarinic receptor–mediated chronotropic responses. A recent study also demonstrated that mice lacking endothelial NO synthase do not exhibit any alterations on cardiac muscle function by muscarinic receptor stimulation, thus refuting the idea that NO plays a role in parasympathetic control.

In summary, in this particular hypertension model we did not observe any changes on the potency or efficacy of β-adrenoceptors and muscarinic receptors. Whether the nonstatistically different decrease in the isoproterenol pEC_{50} might reflect a desensitization of β_{2}-adrenoceptors remains to be further studied.

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