

# Effect of acute nitric oxide synthase inhibition in the modulation of heart rate in rats

A.L. Fellet<sup>1,2</sup>,  
C. Di Verniero<sup>1,2</sup>,  
P. Arza<sup>1,2</sup>, A. Tomat<sup>1,2,3</sup>,  
A. Varela<sup>1,2</sup>, C. Arranz<sup>1,2,3</sup>  
and A.M. Balaszczuk<sup>1,2,3</sup>

<sup>1</sup>Cátedra de Fisiología, Facultad de Farmacia y Bioquímica,  
Universidad de Buenos Aires, Buenos Aires, Argentina  
<sup>2</sup>Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), CONICET,  
Buenos Aires, Argentina  
<sup>3</sup>Instituto Universitario de Ciencias de la Salud (IUCS), Fundación Barceló,  
Buenos Aires, Argentina

## Abstract

### Correspondence

A.L. Fellet  
Cátedra de Fisiología  
Facultad de Farmacia y Bioquímica  
Universidad de Buenos Aires  
(1113) Junín, 956, 7º Piso  
Buenos Aires  
Argentina  
Fax: +54-11-4964-8280  
E-mail: afellet@huemul.ffyb.uba.ar

Research supported by Universidad  
de Buenos Aires, Fundación Barceló  
and IQUIMEFA-CONICET, Buenos  
Aires, Argentina.

Received July 12, 2002  
Accepted January 29, 2003

Acute nitric oxide synthase inhibition with N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) on chronotropic and pressor responses was studied in anesthetized intact rats and rats submitted to partial and complete autonomic blockade. Blood pressure and heart rate were monitored intra-arterially. Intravenous L-NAME injection (7.5 mg/kg) elicited the same hypertensive response in intact rats and in rats with partial (ganglionic and parasympathetic blockade) and complete autonomic blockade ( $38 \pm 3$ ,  $55 \pm 6$ ,  $54 \pm 5$ ,  $45 \pm 5$  mmHg, respectively; N = 9, P = NS). L-NAME-induced bradycardia at the time when blood pressure reached the peak plateau was similar in intact rats and in rats with partial autonomic blockade ( $43 \pm 8$ ,  $38 \pm 5$ ,  $46 \pm 6$  bpm, respectively; N = 9, P = NS). Rats with combined autonomic blockade showed a tachycardic response to L-NAME ( $10 \pm 3$  bpm, P < 0.05 vs intact animals, N = 9). Increasing doses of L-NAME (5.0, 7.5 and 10 mg/kg, N = 9) caused a similar increase in blood pressure ( $45 \pm 5$ ,  $38 \pm 3$ ,  $44 \pm 9$  mmHg, respectively; P = NS) and heart rate ( $31 \pm 4$ ,  $34 \pm 3$ ,  $35 \pm 4$  bpm, respectively; P = NS). Addition of L-NAME (500  $\mu$ M) to isolated atria from rats killed by cervical dislocation and rats previously subjected to complete autonomic blockade did not affect spontaneous beating or contractile strength (N = 9). *In vivo* results showed that L-NAME promoted a tachycardic response in rats with complete autonomic blockade, whereas the *in vitro* experiments showed no effect on intrinsic heart rate, suggesting that humoral mechanisms may be involved in the L-NAME-induced cardiac response.

### Key words

- Nitric oxide
- Nitric oxide synthase
- Autonomic nervous system
- Heart rate
- Heart

## Introduction

Although the role of nitric oxide (NO) in modulating myocardial contractility has been extensively investigated, the effects of NO on heart rate (HR) have received comparatively little attention (1,2). There is evidence that NO plays an obligatory role in cholin-

ergic modulation of automaticity in isolated myocytes (3,4) and that it participates in vagal control of HR in intact mammals (5,6). Some studies have suggested that NO may alter channel mechanisms at the sinoatrial node and other cardiac cells (7,8). However, the cholinergic regulation of NO pathways in cardiac function remains controversial and

the data in the literature are contradictory. Previous investigations have demonstrated that NO donors may directly stimulate the pacemaking activity of the sinoatrial node, independently of the arterial baroreflex (9), and others have reported that the NO pathway may inhibit L-type calcium channel and cAMP-stimulated hyperpolarization-activated current ( $I_f$ ) (10).

Despite intensive research in this area, however, there are many questions about the underlying mechanisms by which NO modulates HR, in particular whether interactions exist between the NO pathways and the autonomic nervous system (ANS) in this situation (11-13).

Given the contradictory data about the relations between ANS and myocardial cell NO in the control of HR, the present study was performed to investigate the effects of acute administration of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) on the modulation of HR in intact rats, in rats submitted to partial and complete autonomic blockade, and in isolated rat atria.

## Material and Methods

Male Sprague-Dawley rats (230 to 260 g body weight) from the breeding laboratories of the Facultad de Veterinaria (Universidad de Buenos Aires, Argentina) were used for each experimental group throughout all experiments. Rats were housed in a humidity- and temperature-controlled environment with an automatic 12/12-h light/dark cycle. Rats were fed standard rat chow from Nutrimentos Purina (Buenos Aires, Argentina) and received tap water *ad libitum* up to the day of the experiments. Animals were used in compliance with the research animal use guidelines of the American Heart Association.

### *In vivo* studies

Rats were anesthetized intraperitoneally (*ip*) with urethane (1.0 g/kg body weight)

(Sigma, St. Louis, MO, USA) and anesthesia was maintained with supplemental *ip* doses throughout the experiment. A tracheotomy was performed and an endotracheal tube (3.5 or 4 mm ID, Portex) was inserted 4 cm into the trachea and secured. After performing the tracheotomy, a polyethylene cannula was inserted into the right and left femoral vein for phenylephrine (PE) infusion and drug administration, respectively. Mean arterial pressure (MAP) was measured directly with a cannula inserted into the right femoral artery and connected to a pressure transducer. The blood pressure signal was measured continuously with a Statham P23 ID pressure transducer (Gould Instruments, Cleveland, OH, USA) and recorded with a polygraph (Physiograph E & M Co. Inc., Houston, TX, USA). HR was derived from the pulsatile pressor signal via tachographic beat to beat conversion with a tachograph preamplifier (577-26 tachometer, Coulbourn Instruments, Inc., Allentown, PA, USA). Body temperature was monitored with a rectal probe and was maintained at  $37.0 \pm 0.5^\circ\text{C}$  with heating lamps. Data were obtained with a data acquisition program (Labtech Notebook, Laboratory Technologies, Wilmington, MA, USA).

### Experimental design

*Protocol I: Effects of L-NAME (7.5 mg/kg) on MAP and HR in intact, ganglionic, vagotomized and anesthetized rats with complete autonomic blockade.* The rats were divided into four groups. Group 1, nine rats with intact ANS and with no previous treatment; group 2, nine rats submitted to ganglionic blockade by administration of an intravenous (*iv*) bolus of 10 mg/kg hexamethonium (Sigma); group 3, nine rats submitted to parasympathetic blockade by bilateral vagotomy at the supraclavicular level; group 4, nine rats submitted to combined hexamethonium administration (10 mg/kg, *iv*) and bilateral vagotomy to guarantee the blockade of

both sympathetic and parasympathetic traffic.

In groups 2 and 4 the administration of hexamethonium was repeated every 20 min to ensure complete blockade of ganglionic transmission during the experiments. Also, PE ( $4\text{--}6\ \mu\text{g kg}^{-1}\ \text{min}^{-1}$ , *iv*; Sigma) was infused during the experiment to maintain MAP within the basal range before L-NAME administration (13,14).

MAP and HR were monitored continuously in all groups. The effects of L-NAME (7.5 mg/kg; Sigma) on MAP and HR were studied by comparing the values obtained 5 min before and after its administration. In addition, in group 4 the pressor and chronotropic responses to L-NAME were also assessed when HR reached the maximum change after L-NAME injection. In additional experiments (9 rats with complete autonomic blockade), a bolus of PE (6  $\mu\text{g/kg}$ , *iv*) was used to determine the specificity of the response to acute L-NAME administration on the cardiovascular parameters.

*Protocol II: Effects of different doses of L-NAME on MAP and HR in rats submitted to complete autonomic blockade.* The pressor response to L-NAME was monitored in rats submitted to complete autonomic blockade using increasing doses of L-NAME, i.e., 5.0, 7.5 and 10 mg/kg,  $N = 9$  per group. The animals were studied after complete autonomic blockade with hexamethonium (10 mg/kg, *iv*) and bilateral vagotomy. The administration of hexamethonium and the infusion of PE were as described in protocol I. MAP and HR were recorded continuously over a period of 60 min after L-NAME administration. Maximum HR and MAP changes after L-NAME injection were used to indicate the magnitude of the response.

#### **In vitro studies**

*Group 1.* Nine male Sprague-Dawley rats (230 to 260 g body weight) were killed by cervical dislocation and the hearts were re-

moved as quickly as possible.

*Group 2.* Before cervical dislocation, nine anesthetized male Sprague-Dawley rats (230 to 260 g body weight) were subjected to complete autonomic blockade as described for the *in vivo* experiments.

The atria from groups 1 and 2 were excised and mounted isometrically under a resting tension of 750 mg in 20-ml organ baths. The bathing medium was a Krebs-Ringer bicarbonate solution,  $\text{pH } 7.40 \pm 0.05$ , of the following composition: 120 NaCl, 25 mM  $\text{NaHCO}_3$ , 4.8 mM KCl, 1.33 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.6 mM  $\text{CaCl}_2$ , 0.02 mM  $\text{CaNa}_2\text{EDTA}$  and 10 mM dextrose. The solution was continuously bubbled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  and the temperature was kept at 31°C. The contraction frequency of spontaneously beating atria was measured from 30-s samples of recorded contractions. After a 60-min recovery period the isolated atria were exposed to L-NAME (500  $\mu\text{M}$ ) and subsequent recordings were obtained at 2, 10 and 30 min.

#### **Statistical analysis**

Data are reported as means  $\pm$  SEM and were analyzed statistically by one-way analysis of variance followed by the Bonferroni test. In some cases, the unpaired Student *t*-test was applied. The 5% probability level was used as a criterion for significance. The Prism software (Graph Pad Software, San Diego, CA, USA) was used for statistical analysis.

## **Results**

#### **In vivo studies**

*Protocol I.* Basal MAP and HR values were similar in all groups of animals. Group 1 =  $83 \pm 3$  mmHg,  $349 \pm 9$  bpm; group 2 =  $82 \pm 4$  mmHg,  $322 \pm 23$  bpm; group 3 =  $88 \pm 7$  mmHg,  $460 \pm 15$  bpm; group 4 =  $83 \pm 6$  mmHg,  $349 \pm 19$  bpm ( $P = \text{NS}$ ).

No differences were observed in the pressor response to a single bolus injection of L-NAME (7.5 mg/kg) in the four experimental groups of animals (Figure 1). The effects of L-NAME on HR in the four experimental groups are shown in Figure 2. L-NAME-induced bradycardia at the time when MAP

reached the peak plateau was of similar magnitude in intact animals and in animals submitted to ganglionic blockade and vagotomy. In contrast, L-NAME administration induced tachycardia after complete autonomic blockade. Moreover, we observed that in group 4 the maximum tachycardic effect was reached

Figure 1. A, Change in mean arterial pressure (MAP) induced by N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). Acute *iv* L-NAME (7.5 mg/kg) significantly increased MAP and this response was similar in intact rats (group 1) and in rats with sympathetic (group 2), parasympathetic (group 3) and complete autonomic blockade (group 4). Data are reported as means  $\pm$  SEM for 9 rats in each group. There were no statistically significant differences between groups (one-way analysis of variance followed by the Bonferroni test). B, Representative tracing of raw data obtained in one experiment for each group showing the effect of L-NAME (7.5 mg/kg, *iv*) on MAP.

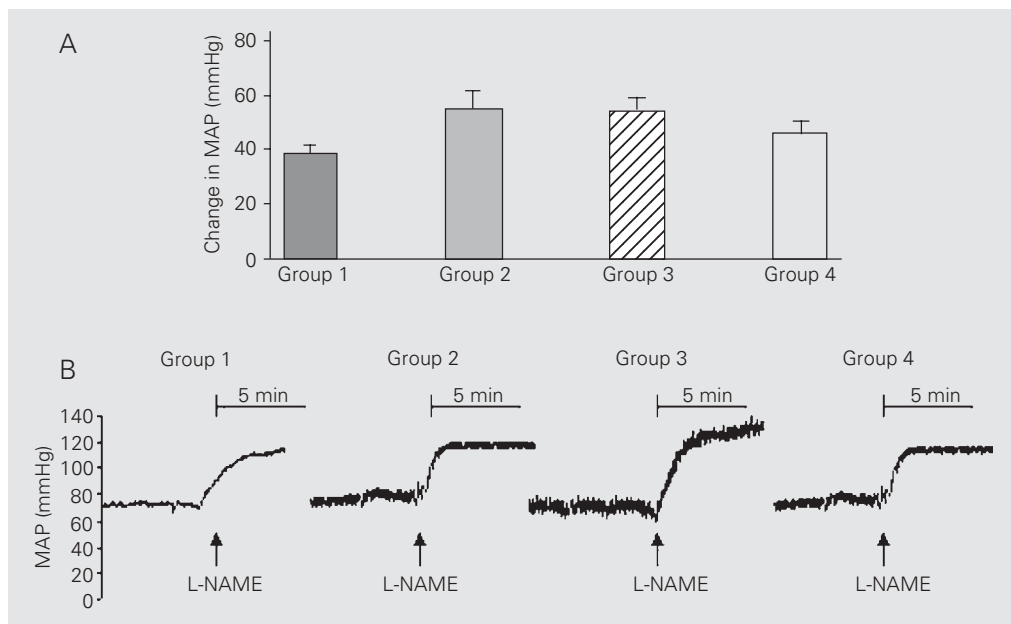
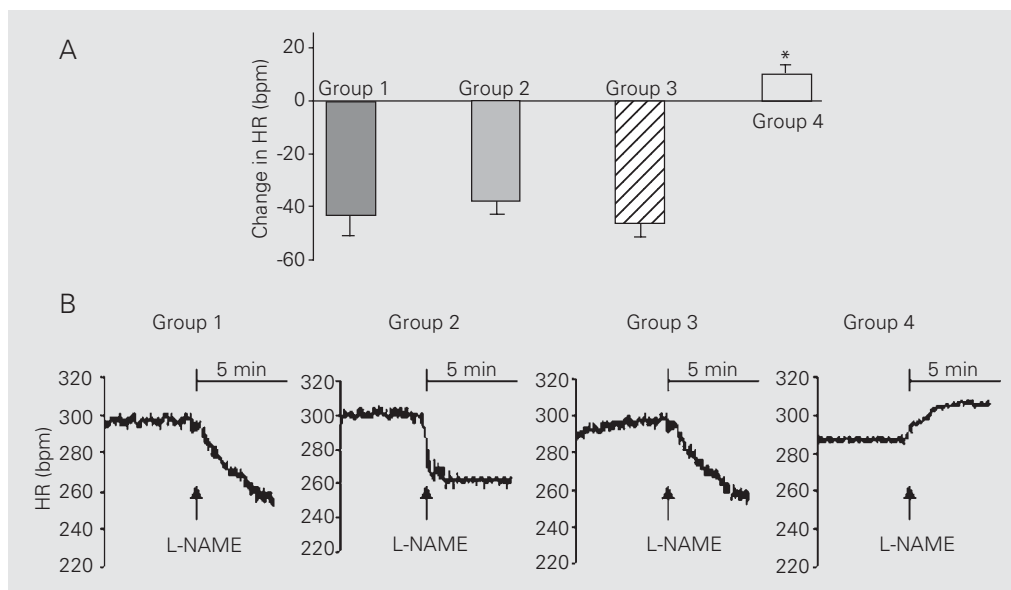


Figure 2. A, Heart rate (HR) response to N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 7.5 mg/kg, *iv*) in intact rats (group 1) and in rats with sympathetic (group 2), parasympathetic (group 3) and complete autonomic blockade (group 4). The L-NAME-induced bradycardia in response to a similar increase in MAP was quantitatively similar in intact, sympathectomized and vagotomized rats. This effect was abolished by complete autonomic blockade. Acute *iv* injection of L-NAME increased HR in group 4 (\* $P$ <0.05 vs group 1, unpaired Student *t*-test). Data are reported as means  $\pm$  SEM for 9 rats in each group. B, Representative tracing of raw data obtained in one experiment for each group showing the effect of L-NAME (7.5 mg/kg, *iv*) on HR.



at about 30 min after *iv* L-NAME administration, from a mean pre-L-NAME value of  $288 \pm 13$  to  $322 \pm 10$  bpm after L-NAME injection ( $P < 0.05$ ).

In completely autonomically blocked rats treated with a bolus injection of PE we observed a similar increase in MAP compared with L-NAME administration in group 4 ( $\Delta$ MAP at 30 min:  $49 \pm 4$  vs  $45 \pm 5$  mmHg, respectively,  $P = \text{NS}$ ). However, HR was unchanged 30 min after PE administration ( $361 \pm 17$  vs  $391 \pm 18$  bpm before PE, respectively,  $P = \text{NS}$ ).

*Protocol II.* Table 1 shows changes in MAP and HR after different doses of L-NAME (5.0, 7.5 and 10 mg/kg) in rats submitted to complete autonomic blockade. There were no significant differences in the magnitude of increase in MAP and HR obtained with the doses tested.

### In vitro studies

The data shown in Table 2 indicate that L-NAME did not affect the pacemaker activity of isolated atria. Table 3 shows that the contractile strength was also unchanged by L-NAME compared to basal values.

## Discussion

*In vivo* protocols were used to study the effects of the NO synthase inhibitor L-NAME on MAP and HR of anesthetized rats submitted to partial and complete autonomic blockade. MAP and HR were similar in all groups of animals at the beginning of the experiments, reflecting similar hemodynamic conditions. A bolus injection of L-NAME (7.5 mg/kg) increased MAP similarly in intact rats and in rats submitted to ganglionic, parasympathetic and complete autonomic blockade. This suggests that the effect of L-NAME administration on blood pressure is independent of sympathetic or parasympathetic influences and is probably due to a direct vasoconstrictor effect caused by the decrease

of vascular NO synthesis. This result agrees with published data demonstrating that the NO-dependent systemic vasodilatation occurs in the absence of a functional ANS (13,15). Furthermore, we have found that at the same time that MAP reached the maximum increase, L-NAME also induced a bradycardic response in intact, vagotomized

Table 1. Effects of increasing doses of L-NAME on changes in mean arterial pressure ( $\Delta$ MAP) and heart rate ( $\Delta$ HR) after complete autonomic blockade.

	L-NAME (mg/kg)		
	5.0	7.5	10.0
$\Delta$ MAP (mmHg)	$45 \pm 5$	$38 \pm 3$	$44 \pm 9$
$\Delta$ HR (bpm)	$31 \pm 4$	$34 \pm 3$	$35 \pm 4$

L-NAME was acute intravenously injected. Data are reported as means  $\pm$  SEM for 9 rats in each group. There were no statistically significant differences among the responses to different doses (one-way analysis of variance followed by the Bonferroni test).

Table 2. Chronotropic response to L-NAME by isolated atria.

	Time (min)			
	Basal	2	10	30
Group 1	$160 \pm 6$	$155 \pm 6$	$158 \pm 7$	$151 \pm 5$
Group 2	$161 \pm 5$	$156 \pm 4$	$153 \pm 5$	$150 \pm 4$

The concentration of L-NAME in the bath was 500  $\mu\text{M}$ . Data are reported as means  $\pm$  SEM bpm for 9 rats in each group. Group 1: cervical dislocation; group 2: complete autonomic blockade + cervical dislocation. There were no statistically significant differences among the responses as a function of time (one-way analysis of variance followed by the Bonferroni test).

Table 3. Inotropic response to L-NAME by isolated atria.

	Time (min)		
	2	10	30
Group 1	$107 \pm 16$	$109 \pm 18$	$109 \pm 20$
Group 2	$108 \pm 18$	$110 \pm 16$	$111 \pm 15$

The concentration of L-NAME in the bath was 500  $\mu\text{M}$ . Data are reported as means  $\pm$  SEM for percent of the basal peak developed tension recorded at the end of the stabilization period.  $N = 9$  rats per group. Groups 1 and 2 are the same as in Table 2. There were no statistically significant differences among the responses as a function of time (one-way analysis of variance followed by the Bonferroni test).

and ganglionic blockaded animals. In contrast, a significant increase in HR was observed in rats with total autonomic blockade. We observed that PE did not induce any increase in HR, confirming the fact that only L-NAME caused an increase in HR in complete autonomic blockaded animals. Since the abolition of baroreflex function in rats with complete autonomic blockade is not the cause of L-NAME-induced tachycardia, we were able to assess the chronotropic response to L-NAME independently of the baroreflex response.

The rise in blood pressure that activates baroreceptor afferents results in both direct and indirect activation of the parasympathetic vagal innervations of the heart, decreasing HR in intact and ganglionic blockaded rats. When both vagus nerves are sectioned, the bradycardic response still occurs and this chronotropic response may be due to the inhibition of sympathetic tone (16).

Focusing on the immediate L-NAME-induced tachycardia, we determined whether this chronotropic effect persisted in the 5- to 10-mg/kg dose range of the NO synthase inhibitor. The results showed that the different doses of L-NAME used caused a similar increase in MAP and HR in rats with complete autonomic blockade. The tachycardic effect of L-NAME on these animals was perhaps surprising. Other studies have shown contradictory results with respect to the chronotropic effects of NO synthase inhibition (17) and the antimuscarinic properties of L-NAME in *in vivo* experiments (18). The mechanism responsible for the L-NAME-induced tachycardia in rats with autonomic blockade is unclear. The results obtained in the present study, which disagree with others (9,10,13,18) may be due, at least in part, to differences in some methodological conditions and/or to differences in the autonomic tone of the animals.

NO synthase has been shown to be present in many sites of the central and peripheral nervous system and is involved in the

autonomic control of cardiovascular function (19,20). Recent studies have indicated that changes of NO in the central nervous system may contribute to the regulation of the cardiovascular system by inhibition of the sympathetic nervous system and modulation of the arterial baroreflex (21). Additionally, previous reports have demonstrated that 30 to 60 min are required for *iv* L-NAME to cross the blood-brain barrier and reach a sufficient concentration in the central nervous system to inhibit more than 50% of central neuronal NO synthase activity in the brain (22,23). The chronotropic response induced by L-NAME reached a maximum value at about 30 min after L-NAME administration, suggesting that the activation of humoral mechanisms (endothelins, angiotensin II, atrial natriuretic peptide, thyroid hormones and others) as well as physical factors such as shear stress or a direct effect of L-NAME may be probably involved in the HR response (24-26).

Thus, based on our findings in *in vivo* experiments, we evaluated the effect of L-NAME on pacemaker and contractile activity in *in vitro* studies. To assess the possible similarity of autonomic conditions between *in vivo* and *in vitro* experiments, a complete autonomic blockade was induced before cervical dislocation. The data showed that the addition of L-NAME at a dose equivalent to that used *in vivo* did not modify the chronotropic or inotropic effects under our experimental conditions. The L-NAME-induced tachycardia observed in *in vivo* experiments may not be due to a direct action of the inhibitor on the pacemaker. In isolated right atria, Riado et al. (27) showed that L-NAME did not affect the beat rate, whereas others have reported that low doses of an NO donor or 8-bromo-cGMP increase the spontaneous beating of isolated guinea pig atria and rabbit sinoatrial node cells by activation of the hyperpolarization inward current (28,29). In addition, NO can elicit either positive or negative inotropic effects (30,31). At pres-

ent, it is unclear how the intrinsic rate depends on NO production within cardiac cells and many *in vitro* and *in vivo* studies have illustrated the multiple and sometimes contradictory effects of NO pathways on cardiac function.

We have shown that: a) an acute L-NAME bolus of 7.5 mg/kg induced the same pressor response in all experimental groups of animals, and this hypertensive effect of L-NAME was independent of the ANS; b) acute L-NAME administration to intact, vagotomized and ganglionic blocked rats promoted bradycardia associated with an increase in blood pressure; c) *iv* injection of L-

NAME in rats with complete autonomic blockade induced an immediate positive chronotropic effect; d) L-NAME administration had no direct effect on the spontaneous beating or contractile activity of *in vitro* isolated atria; e) humoral and physical factors may be probably involved in the L-NAME-induced response.

### Acknowledgments

The authors wish to thank Ana María Bento Schneider, Associate Teacher of English at the School of Medicine, Universidad de Buenos Aires, Argentina.

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