

# The Effects of Oral Arginine on Neuroautonomic Parameters in Healthy Subjects

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## ABSTRACT

L-arginine is an essential amino acid that exerts both peripheral and central nervous system effects. Although it has been used as a growth hormone releasing agent, this amino acid also provokes significant blood pressure and heart rate changes in all patients tested. This study assessed the changes induced by the oral administration of this amino acid on the peripheral autonomic nervous system in 52 subjects (26 L-arginine, 26 placebo). The levels of the following circulating neurotransmitters were measured before and after a small oral dose (50 mg) of L-arginine: noradrenaline (NA), adrenaline (Ad), dopamine (DA), plasma serotonin (f-5HT), platelet serotonin (p-5HT), and plasma tryptophane. Systolic blood pressure, diastolic blood pressure, and heart rate were also monitored. L-arginine triggered sustained and progressive increases of NA, DA, f-

5HT, p-5HT, and the f-5HT/p-5HT ratio as well as sustained and progressive decrease of Ad. Diastolic blood pressure but not systolic blood pressure showed significant and progressive reductions. Progressive heart rate reductions were also observed. Significant positive correlation was registered between diastolic blood pressure versus NA/DA ratio and significant negative correlation was found between heart rate versus f-5HT/p-5HT ratio. An oral administration of 50 mg of L-arginine was able to provoke parasympathetic over sympathetic and neural-sympathetic over adrenal-sympathetic predominance.

## INTRODUCTION

Nitric oxide (NO) is synthesized from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS), and it modulates a wide variety of neural, cardiovascular, endocrinologic, and humoral processes. Cardiovascular autonomic dysfunction and impaired neurohormonal secretion are characteristics often seen in patients with primary autonomic failure and, in addition, are also involved in blood pressure and heart rate disorders. NO, which plays an important role

in the balance of the peripheral autonomic nervous system (ANS) by acting at both peripheral and central nervous system (CNS) levels, is likely to be involved in all of the above-mentioned disorders. For instance, abrogation and enhancement of NO activity are associated with sympathetic and parasympathetic predominance, respectively; both ANS imbalances are present in all diseases. In addition, it has been shown that nitrergic pathways are involved in serotonergic and cholinergic mechanisms responsible for vascular physiologic modulations.<sup>1</sup>

According to the above and considering that L-arginine has been used as a coadjuvant therapeutic tool in different types of diseases, the purpose of this study was to investigate the neuroautonomic mechanisms triggered by a small oral dose of L-arginine that was administered to subjects. This research was possible because the neurochemical laboratory at the Instituto de Medicina Experimental, Faculty of Medicine at the Universidad Central de Venezuela has the capability to assess all circulating neurotransmitters, and this is where those parameters were routinely tested before and after the administration of many neuropharmacologic agents,<sup>2-7</sup> as well as during supine-resting plus orthostasis plus exercise and several pharmacologic challenges.<sup>8-17</sup> Up to the present, approximately 30,000 healthy and diseased subjects as well as many experimental mammals have been tested. The protocol included the assessment of noradrenaline (NA), adrenaline (Ad), dopamine (DA), platelet serotonin (p-5HT), plasma serotonin (f-5HT), and plasma tryptophane (trp). In addition, the metabolites of these neurotransmitters were tested according to different types of protocols. The results obtained from an oral dose (50 mg) of L-arginine, which is the dose usually administered to patients in order to excite growth

hormone release at nocturnal periods, is presented. However, in this study, the effects of this amino acid were obtained throughout the supine-resting condition during morning periods.

## **SUBJECTS AND METHODS**

The levels of plasma NA, Ad, DA, f-5HT, p-5HT, and plasma trp were measured before (-30 and 0 minutes) and after (60, 90, and 120 minutes) the oral administration of 50 mg of L-arginine (Ajinomoto Co., Inc., Tokyo, Japan) in 26 subjects (13 men and 13 women). Twenty-six other age- and sex-paired subjects received placebo instead of the drug. Ages ranged from 22 to 29 years (mean  $\pm$  standard error was  $25.6 \pm 2.2$ ). Platelet aggregation was also measured. All subjects were volunteers recruited from the students of the Faculty of Medicine at the Universidad Central de Venezuela. Written informed consent was obtained from all volunteers, and the procedure was approved by the Ethical Committee of FUNDAIME (Fundación Instituto de Medicina Experimental). All volunteers were within 10% of ideal body weight, none had any physical or psychiatric illness, and all were nonsmokers. Exclusion criteria included pregnancy, lactation, and alcohol abuse.

Volunteers were recumbent during all procedures. A heparinized venous catheter was inserted into a forearm vein at least 30 minutes before the test. Cold plastic syringes were used to collect blood samples at the times specified above. L-arginine (50-mg capsule) or placebo was orally administered after the second blood sample (0 minutes). Blood samples were obtained for measuring plasma neurotransmitters and platelet aggregation. Blood for measuring plasma neurotransmitters was transferred to plastic tubes, each containing 1 mL of an antioxidant solution (20 mg of ethylenediaminetetraacetic acid [EDTA]

plus sodium metabisulfite 10 mg/mL). The tubes were carefully inverted several times and placed on ice until centrifugation. To obtain platelet-rich plasma (PRP), the tubes were centrifuged at 600 rpm at 4°C for 15 minutes. Two milliliters of PRP was stored at -70°C until needed for determination of p-5HT levels. The remaining blood was centrifuged again at 7000 rpm. Two aliquots of the supernatant, which was platelet-poor plasma (PPP), was stored at -70°C until needed for assays of catecholamines and f-5HT. Blood samples for platelet aggregation were processed immediately. A physician was in constant attendance who monitored heart rate and blood pressure and noted any symptoms reported by subjects.

### **Analytical Assays**

#### ***Neurochemistry***

Plasma catecholamine and serotonin samples were measured in duplicate, and all determinations were made at the same time. Reverse phase, ion pair high-pressure liquid chromatography with electrochemical detection was used.<sup>18-20</sup>

#### ***Reagents and Standards***

NA, Ad, DA, serotonin creatinine sulfate, dihydroxybenzylamine, 5-hydroxytryptophane, sodium octyl sulfate, dibutylamine  $\text{KH}_2\text{PO}_4$ , citric acid, sodium acetate, and EDTA were obtained from Sigma-Aldrich Co. (St. Louis, MO). Acid-washed aluminum oxide and microfilters were purchased from Bioanalytical Systems Inc. (West Lafayette, IN). Acetonitrile and 2-propranol were obtained from Riedel-deHaen AG (Frankfurt, Germany). Glass-distilled water was de-ionized and filtered through a Milli-O reagent grade water system (Millipore, Bedford, MA). Solutions and solvent were filtered through a 0.2  $\mu\text{m}$  Millipore filter and were vacuum de-aerated. Standard solutions (1 mmol/L) were prepared in

0.1 mol/L perchloric acid and diluted to the desired concentration.

#### ***Equipment***

Liquid chromatography was performed using Waters 515 pumps (Waters Co., Milford, MA) equipped with 7125i Rheodyne valve injector fitted with a 20-ML sample loop for detection of catecholamines, and 50- $\mu\text{L}$  sample loop for p-5HT and f-5HT detection (Rheodyne, Berodine, Berkeley, CA). For catecholamine assays, a 15 cm x 4 mm ID Discovery (Supelco, Sigma-Aldrich Co.) analytical column packed with C18 3  $\mu\text{m}$  particles was used, fitted with a precolumn filter 0.2  $\mu\text{m}$  (Sigma-Aldrich Co.).

The detection system was a Waters 460 Electrochemical Detector (Waters Co.). A potential of 0.70 volts was applied to the working electrode (glassy carbon) versus the silver-silver chloride reference electrode. The chromatograms were registered and quantified using Millennium software (Waters Assoc., Milford, MA).

#### ***Catecholamine Assay***

These were performed by extraction onto 20 mg of acid washed alumina followed by their elution with 200  $\mu\text{L}$  of 0.2 mol/L perchloric acid using Bioanalytical Systems microfilters. The instrument was calibrated with standard plasma. After incubation with acid-washed aluminum oxide, a plasma-pool free of catecholamines was obtained. This was processed similarly to plasma samples, but 20  $\mu\text{L}$  of standard solution containing NA, Ad, and DA (50 ng/mL each) was added to 1 mL of the plasma pool to obtain the standard plasma. Both standard plasma and sample plasma were supplemented with 20  $\mu\text{L}$  of internal standard solution (dihydroxybenzylamine 100 ng/mL). The mobile phase was composed of  $\text{KH}_2\text{PO}_4$  50 mmol/L, EDTA 25.16 nmol/L, sodium octyl sulfate 2.37 mmol/L, di-N-buty-

lamine 100 µL/L, and acetonitrile 2.5% (v/v) with pH adjusted to 5.2.

Catecholamine determinations were performed after injection of 50 µL of processed plasma. Correction for dilution was performed. Concentrations were expressed in pg/mL. The sensitivity of this method is as follows: NA was 3.2 pg/mL, Ad was 4.2 pg/mL, and DA was 2.5 pg/mL. The intra-assay coefficients of variation were 2.3%, 3.6%, and 2.3% for NA, Ad, and DA, respectively. The inter-assay coefficients of variation were 2.6%, 3.9%, and 3.8%, respectively.

### **Serotonin Assay**

After sonication of PRP to disrupt any intact platelets (Ultrasonic Liquid Processor, model 385, Heat Systems Ultrasonic, Inc., Farmingdale, NY), both PRP and PPP were processed in the same way: 200 µL of 3.4 M of perchloric acid as deproteinizing agent and 10 µL of 5-OH-trp solution (80 µg/mL) as internal standard, were added to 1 mL of plasma, vortexed, and centrifuged at 10,000 rpm x 15 minutes at 4°C. The clear supernatant was filtered through a 0.22-µm membrane (Millipore) and injected in high-pressure liquid chromatography. Calibration runs were generated by spiking blank plasma containing 50 µL of serotonin solution (10 µg/mL) and 10 µL of 5-OH-trp solution (80 µg/mL). This standard plasma was processed in the same manner as the samples. PRP serotonin (p-5HT) and PPP serotonin (f-5HT) levels were determined after injection of 100 µL of the deproteinized sample onto a 30 cm x 4.0 mm ID Discovery column filled with C18 5µm. The mobile phase was composed of citric acid 20 mmol, sodium acetate 50 mmol, sodium octyl sulfate 6.45 nmol, dibutylamine 100 µL/L, and propanol 3.5% (v/v). Ph was adjusted to 4.9; flow rate was adjusted to 0.70 mL/min. The sensitivity of this method for plasma serotonin was 0.18 ng/mL; intra-assay

coefficients of variation were 2.8% for PRP serotonin and 3.1% for PPP serotonin. Inter-assay coefficients of variation were 3.5% and 5.2%, respectively. Correction factor for dilution was used. Concentrations are expressed in ng/mL. Platelet serotonin value (p-5HT) = PRP serotonin value (total circulating serotonin) minus PPP serotonin value (f-5HT).

### **Platelet Aggregation**

Blood was collected with citrate-phosphate dextrose (1:9 v/v) as the anticoagulant. Blood was subsequently centrifuged at 120 g for 10 minutes to prepare PRP. Aggregation studies were carried out according to Born's method,<sup>21</sup> and aggregation was induced by adenosine 5'diphosphate (Fluka, Sigma-Aldrich Co.) and collagen at final concentrations of 4 µmol/L and 4 µg/mL, respectively. Maximal aggregation, expressed as the percentage of maximal light transmission, was measured.

### **Statistical Analyses**

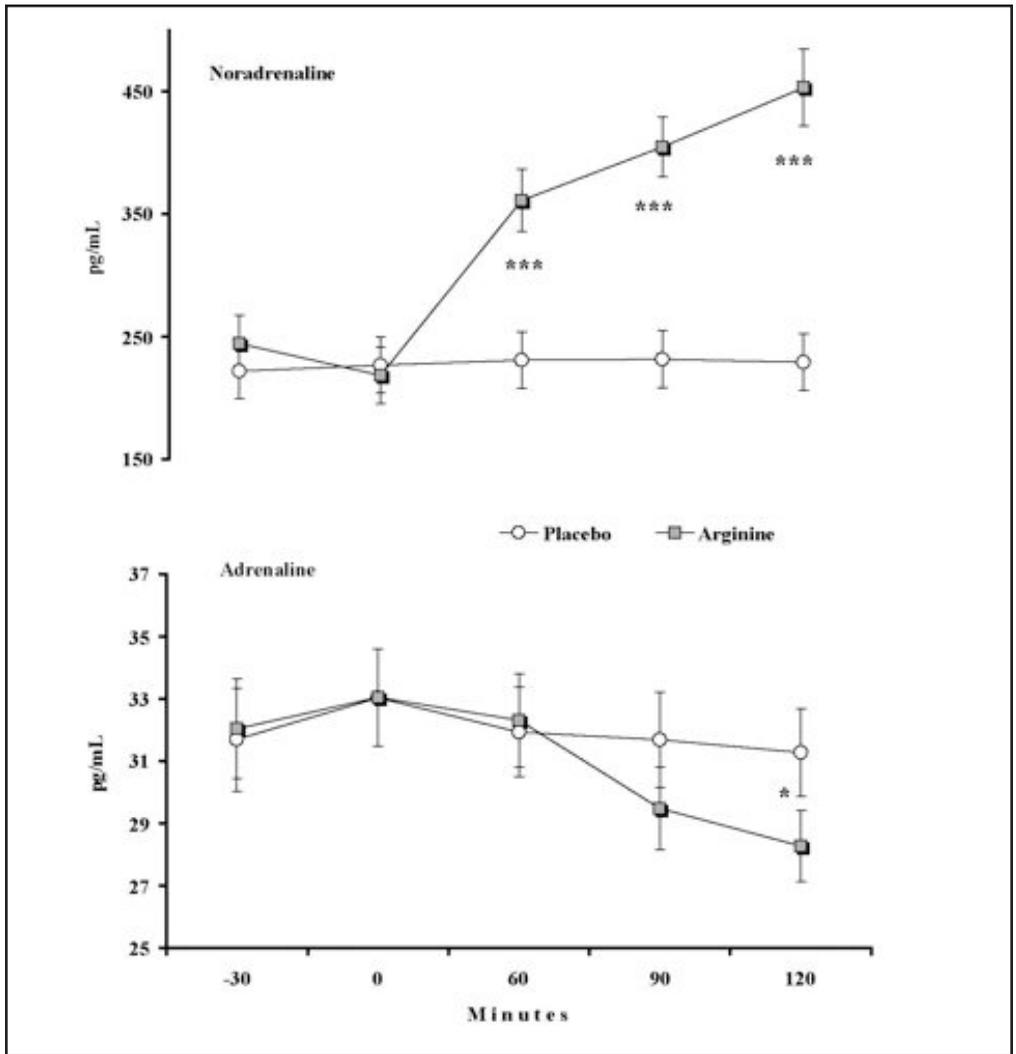
Results were expressed as mean ± SE. Multivariate analyses of variance with repeated measurements, paired *t*-test, and correlation coefficients (exploratory factor analysis) were employed in interpreting the data yielded by this investigation. Differences were considered significant at  $P \leq 0.05$ . Stat-100 General Statistics Package (Biosoft, Great Shelford, Cambridge, UK) was used for statistical analyses.

## **RESULTS**

### **Catecholamines**

NA was significantly and progressively increased at all periods following L-arginine administration. Maximal increases in plasma NA occurred at the 120-minute period (Figure 1).

DA also showed significant and progressive increases. Maximal increase occurred at the 120-minute period



**Figure 1.** L-arginine (50 mg orally) triggers the increase and decrease of plasma noradrenaline and adrenaline, respectively. Mean  $\pm$  standard error. (\*)  $P < 0.05$ ; (\*\*\*)  $P < 0.005$ .

(Figure 2).

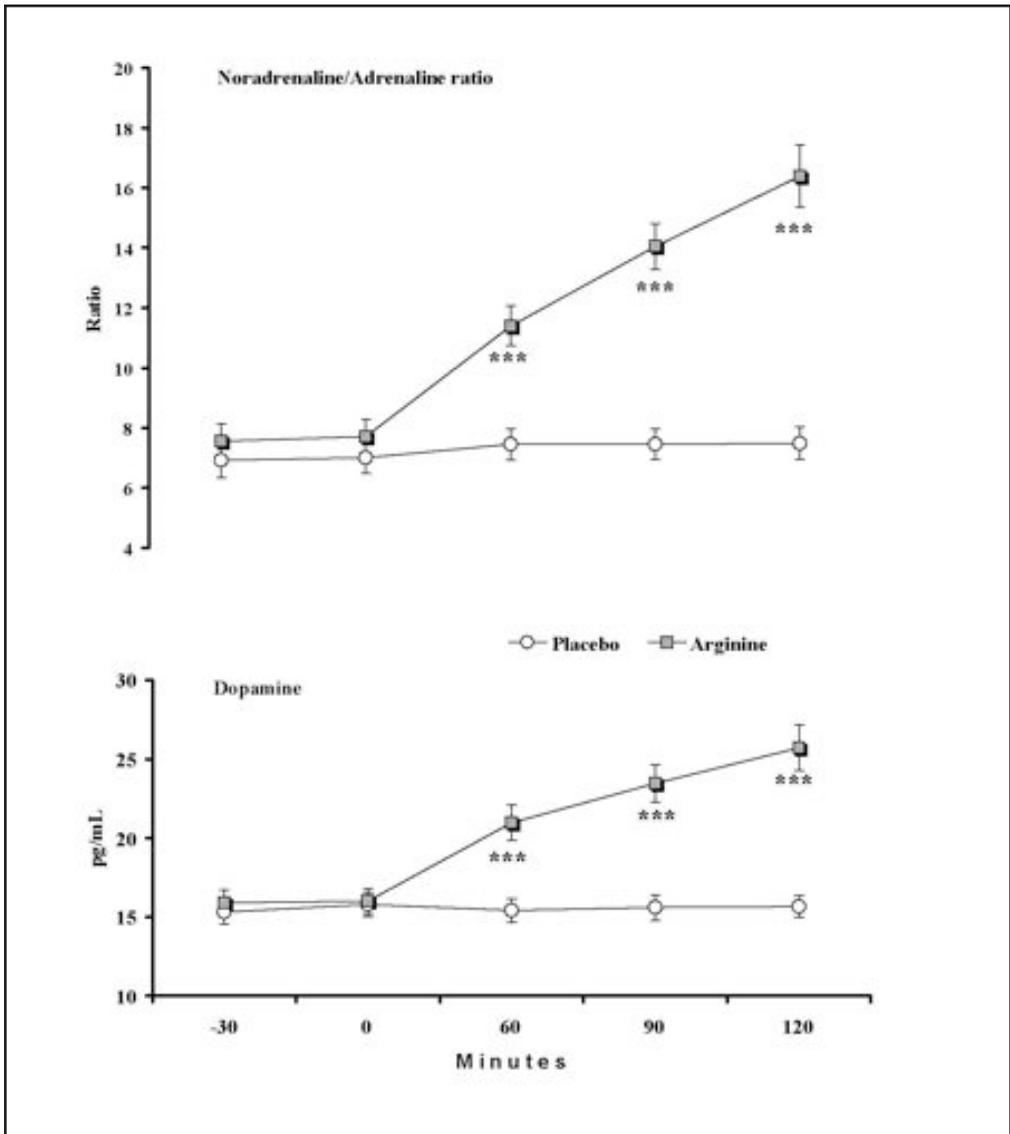
Ad showed slight and progressive decrease (significant at the 120-minute period) (Figure 1).

### Indoleamines

p-5HT showed slight but sustained and significant increases throughout all post-arginine periods (Figure 3). f-5HT showed great and progressive increases until it reached maximal rise at the 120-

minute period. These rises overwhelmed the p-5HT increases in such a way that the f-5HT/p-5HT ratio showed progressive and significant augmentation (Figure 3).

Both the NA increase and the Ad decreases resulted in a progressive NA/Ad ratio rise which reached maximal values at the 120-minute period (Figure 2).



**Figure 2.** L-arginine (50 mg orally) triggers the increase of both noradrenaline/adrenaline ratio and plasma dopamine. Values are expressed as mean  $\pm$  standard error. (\*\*\*)  $P < 0.005$ .

### Blood Pressure

The significant and progressive reduction of the diastolic blood pressure (DBP) but not the systolic blood pressure (SBP) resulted in a progressive and significant rise of differential blood pressure (SBP-DBP) (Figure 4).

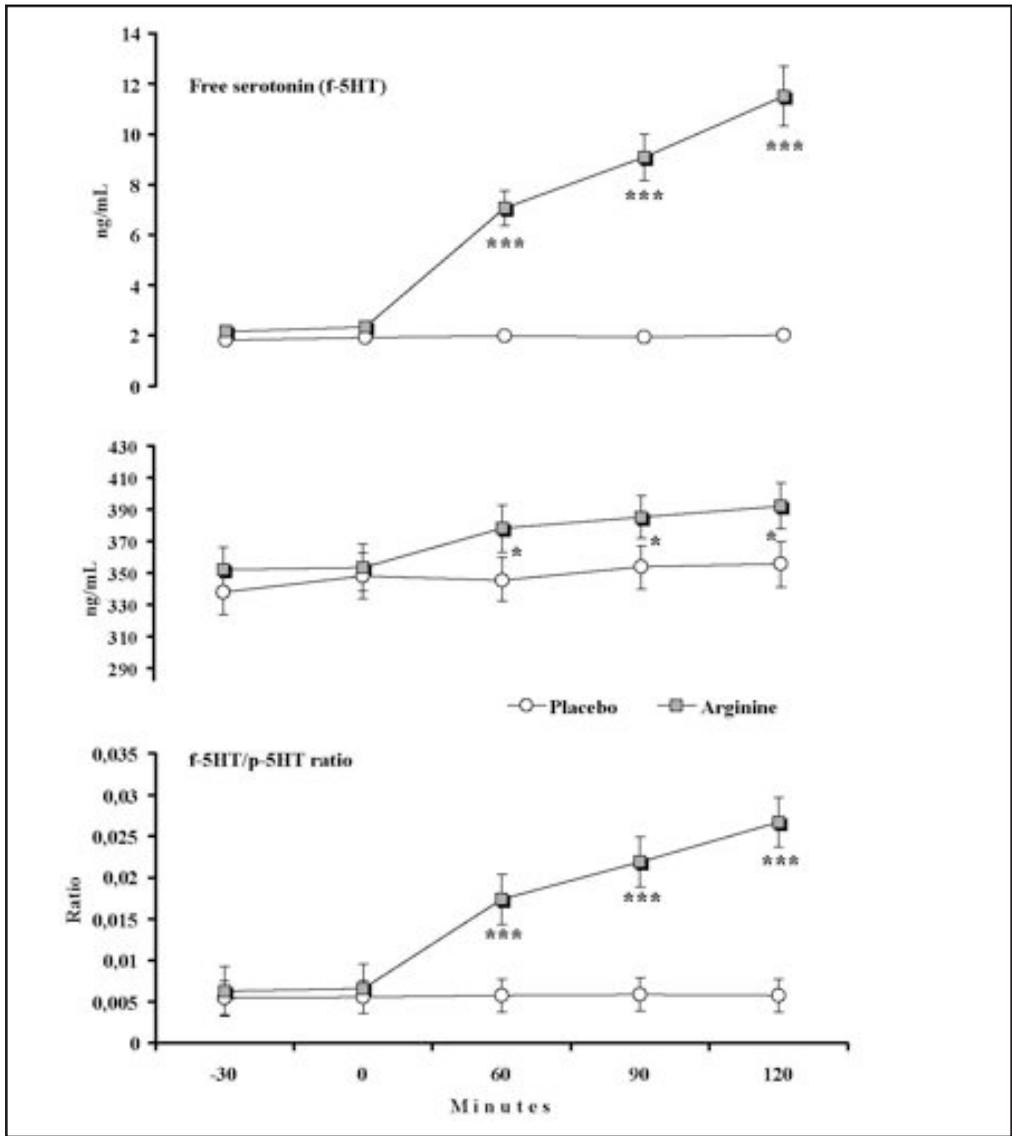
### Heart Rate

This parameter showed significant and

progressive reductions (Figure 4), which paralleled DBP decreases.

### Correlations

Significant positive correlations were found between NA/DA ratio versus DBP values:  $r=0.53, 0.61, 0.68$ ;  $P < 0.05, P < 0.02, P < 0.01$  at 60-, 90-, and 120-minute periods, respectively, whereas significant negative correlations were



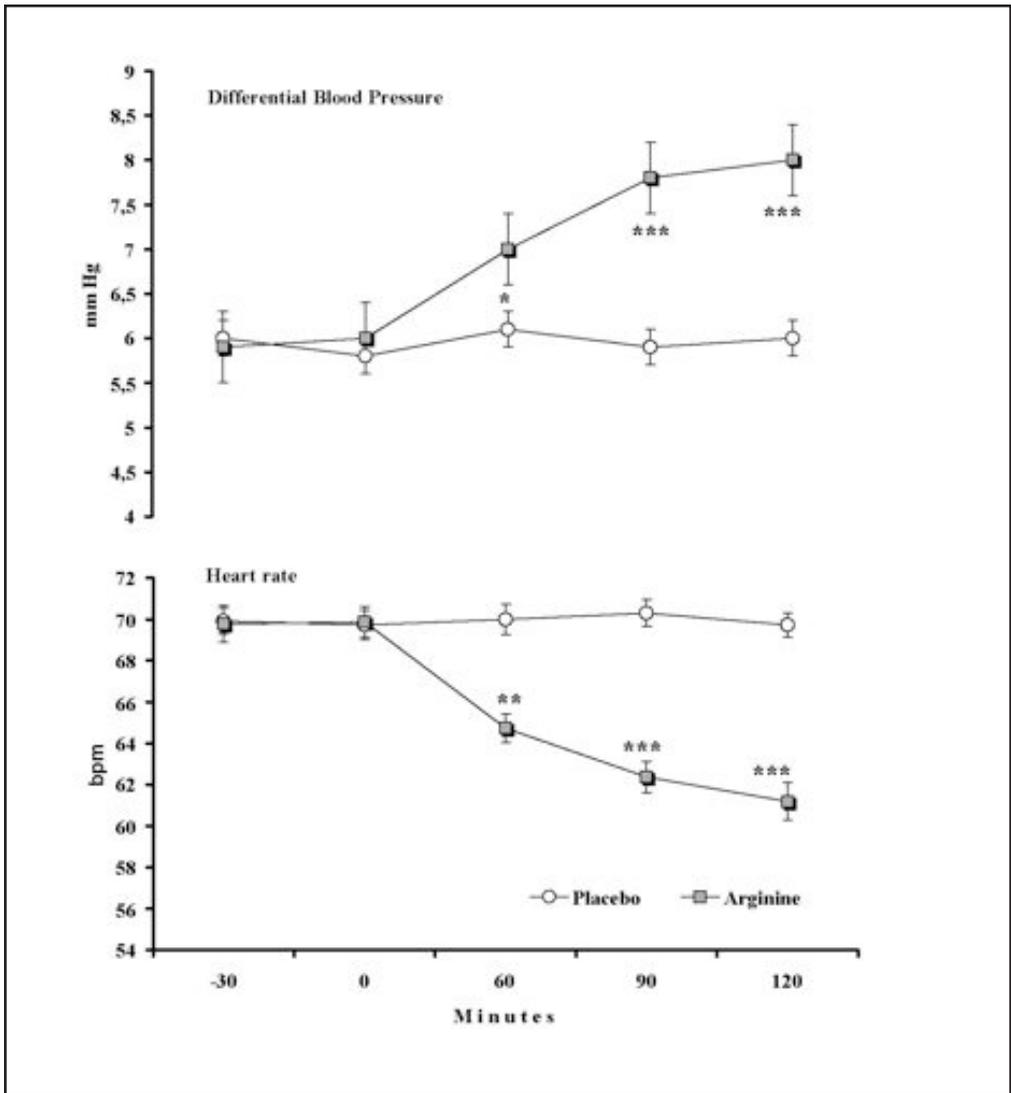
**Figure 3.** L-arginine (50 mg orally) provokes the increase of plasma serotonin (f-5HT), platelet serotonin (p-5HT), and the f5HT/p5HT ratio. Mean  $\pm$  standard error. (\*)  $P < 0.05$ ; (\*\*\*)  $P < 0.005$ .

registered when tested NA/Ad ratio versus f-5HT/p-5HT ratio:  $r = -0.51, -0.69, -0.73$ ;  $P < 0.05, P < 0.01, P < 0.005$ . In addition, heart rate decreases correlated negatively with f-5HT/p-5HT ratio:  $r = -0.56, -0.71, -0.77$ ;  $P < 0.05, P < 0.01, P < 0.005$  at 60-, 90-, and 120-minute periods, respectively. Significant positive correlations were found when DBP was compared

with NA values:  $r = 0.47, 0.59, 0.67$ ;  $P < 0.05, P < 0.02, P < 0.01$ , as well as significant negative correlations when DBP was compared with DA values:  $-0.46, -0.53, -0.56$ ;  $P < 0.05, P < 0.05, P < 0.02$  at 60-, 90-, and 120-minute periods, respectively.

**DISCUSSION**

The results obtained from this study



**Figure 4.** L-arginine (50 mg orally) triggers the increase and decrease of differential blood pressure (SBP-DBP) and heart rate, respectively. Mean  $\pm$  standard error. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.005$ .

show that a small dose (50 mg) of oral L-arginine was enough to induce significant changes in the levels of circulating neurotransmitters, DBP, and heart rate in healthy subjects. These physiologic changes were paralleled by feelings of well-being and mental brightness, which might be attributed to the enhancement of neural sympathetic activity, as revealed by the rise of the NA but not Ad plasma levels, together with the

increase of parasympathetic tone, as revealed by the neurophysiologic, neurochemical, and clinical parameters.

Although a bulk of research work has been devoted to the effects of L-arginine on mammals, most if not all of these reports are based on the administration of high doses (oral or parenteral) of this amino acid, which obviously provoke complex responses that are hard to understand and associate with their inti-

mate underlying physiologic mechanisms. In addition, most research dealing with the effects triggered by this amino acid was performed to show singular effects obtained from lineal experiment design; however, those experimental findings allowed a better understanding of the results of this study.

L-arginine is a NO releasing agent whose inhibition is associated with arterial hypertension and the enhancement of sympathetic activity.<sup>22</sup> These effects are paralleled by deficits of NO at the hypothalamic paraventricular nucleus (PVN).<sup>23</sup> In addition, it has also been reported that angiotensin is involved in the hypertensive mechanisms associated with the inhibition of NO.<sup>24</sup> The CNS-mediated sympatho-inhibition triggered by NO would involve NOS (neuron-mediated) mechanisms including CNS serotonergic (5-HT) nuclei.<sup>25</sup> At the same time, other research work shows that the NO synthesis inhibition triggers adrenal sympathetic hyperactivity plus neural sympathetic inhibition; this is the opposite of the ANS profile that was reported in this study.<sup>26</sup> Other experimental findings show that DBP was reduced by L-arginine (30 mg/kg IV) because it facilitates NO synthesis at the CNS level.<sup>27</sup>

Other findings show that the inhibition of CNS-NO synthesis triggers hypertension because it increases adrenal sympathetic activity.<sup>28</sup> These findings support the results of this study, which show that L-arginine annuls adrenal sympathetic activity. Many other research studies report that CNS mechanisms are involved in the L-arginine-NO sympathetic modulatory role. With respect to this, it has been shown that the glutamic acid N-methyl d-aspartate receptor mediates mechanisms that are responsible for the adrenal sympathetic hyperactivity associated with NO-synthesis inhibition. This phenomenon includes the PVN hypothalamic nucleus<sup>29</sup> and the dorsomedial and rostral

ventrolateral medulla<sup>30</sup> as well as the medullary nucleus of the solitary tract (NTS).<sup>31</sup> These findings are supported by others that show that NO synthesis in the brain inhibits the Ad release from the adrenal glands.<sup>32</sup> All these findings are consistent with the results of this study which show the reduction of Ad plus the increase of the NA/Ad ratio after oral L-arginine administration. Kimber et al<sup>33</sup> reported that L-arginine increased DBP in patients affected by postural and postprandial hypotension, which is due to a deficit of neural sympathetic activity. Conversely, Prados and colleagues<sup>34</sup> associated the deficiency of L-arginine with the age of spontaneously hypertensive rats, which could be linked to the inefficient production of NO. Similar postulation is presented by Augustyniak et al<sup>35</sup> who suggested the inclusion of NO-excitatory mechanisms in order to attempt new therapeutic strategies for patients with hypertension. Furthermore, findings by Kvetnansky and colleagues<sup>36</sup> reinforce the above postulation with the demonstration that the blockade of NO synthesis with N-nitro-L-arginine (L-NAME) triggers adrenal sympathetic activation and arterial hypertension, as reflected by the reduction of the NA/Ad ratio, the opposite catecholaminergic profile to that registered in the present study after the administration of oral L-arginine.

The findings by Claxton and Brands<sup>37</sup> are in agreement with all of the above. Their work shows that the NO-synthesis inhibition with L-NAME is responsible for the hypertension triggered by glucose infusion associated with the suppression of the neural sympathetic activity plus the enhancement of adrenal sympathetic drive.

Other findings by Giugliano et al<sup>38</sup> show that L-NAME administration in humans induces vasodilation and platelet aggregation, which is associated with Ad release. The aforementioned

research studies support the findings of this study by showing that L-arginine administration enhances parasympathetic activity. The latter should be associated to the overproduction of NO at the CNS level. With respect to this, the excitation of the NTS parasympathetic medullary nucleus plus the inhibition of the C1-Ad rostral ventrolateral medullary nuclei through the L-arginine-NO pathway has been found to play a key role into this mechanism.<sup>39</sup> In addition, other research reports that L-arginine uptake is stimulated by acetylcholine in vivo and in vitro.<sup>40</sup>

The fact that L-arginine lowers DBP despite the increase of neural sympathetic activity (NA + DA), as seen in this study, is explained not only because the inhibition of the adrenal glands provoked by the drug but also by the reduction of the NA/DA ratio. This is because the direct vasodilator effect was triggered by NO at the vascular area, at which level NO was able to deactivate the vasoconstrictor effect of NA.<sup>41</sup> Thus, stimulation of the endogenous NO pathway would enhance parasympathetic activity against the influences of NA-sympathetic effect, as shown by Chowdhary et al<sup>42</sup> in humans. The information mentioned above has been summarized by Lepori and colleagues,<sup>43</sup> and is consistent with the postulation that acetylcholinergic mechanisms play an important hitherto unrecognized role in offsetting the hypertension and cardiac sympathetic activation caused by NOS inhibition in humans.

Decreased parasympathetic activity and impaired NO synthesis would characterize several cardiovascular disease states, including hypertension. With respect to this, the findings by Prior et al<sup>44</sup> show that short-term oral supplementation with L-arginine produced marked and sustained elevation of acetylcholine and vasodilation are in agreement with the above-mentioned research. Finally,

Elayan and colleagues<sup>45</sup> report that the inhibition of L-arginine synthesis with L-NAME enhances adrenal sympathetic activity, which is the opposite phenomenon seen in the present study.

Other additional studies reinforce these results. For instance, acetylcholine enhances the uptake of L-arginine both in vitro and in vivo.<sup>40</sup> This is consistent with the findings of the present study that show the lowering of DBP and heart rate by L-arginine.

The NA/Ad ratio increase triggered by the small dose of oral L-arginine registered in this study supports the postulation that neural but not adrenal sympathetic activity was enhanced by the drug. The fact that adrenal glands secrete 80% of Ad whereas sympathetic nerves release 80% to 90% of NA plus 10% to 20% of DA are consistent with all the above. The close positive correlations shown between the 2 latter parameters but not with Ad reinforce the postulation. NA versus DA:  $r=0.63, 0.69, 0.65; P<0.01$  in all cases.

The dissociation between both adrenal and neural sympathetic activities triggered by L-arginine would explain why the DBP but not the SBP dropped throughout the present trial. With respect to this, it should be remembered that a DA pool exists at sympathetic nerves which is released before NA and modulates its release by acting at presynaptic DA-2 receptors.<sup>46,47</sup> This postulation is supported by the close DA versus DBP negative correlations registered in this study:  $r=-0.46, -0.53, -0.56; P<0.05, P<0.05, P<0.02$  at 60-, 90-, and 120-minute periods, respectively. These significant negative correlations become positive when DBP values were plotted against NA/DA ratio:  $r=0.53, 0.61, 0.68; P<0.05, P<0.02, P<0.01$  at 60-, 90-, and 120-minute periods, respectively.

Two mechanisms might explain the reduction of heart rate provoked by L-arginine in the subjects in the present

study: a) the reduction of the adrenal sympathetic activity and b) the enhancement of parasympathetic activity. The significant positive Ad versus heart rate and negative heart rate versus the f-5HT/p-5HT ratio fit well with the authors' postulation. In effect, it has been shown that f-5HT competes with circulating acetylcholine for the platelet uptake,<sup>48</sup> thus the increase of the f-5HT/p-5HT is registered during parasympathetic enhancements. This phenomenon is consistent with the fact that atropine provokes an abrupt reduction of the f-5HT/p-5HT ratio in both normal and hyper-parasympathetic syndromes (Bezold-Jarisch reflex).<sup>49-55</sup>

The above hyperparasympathetic activity is explained because plasma serotonin excites the medullary area postrema located outside the blood brain barrier and activates parasympathetic mechanisms that increase the release of serotonin from the enterochromaffin cells, associated with the blood pressure and heart rate decreases.<sup>51,56-59</sup> The increase of circulating serotonin triggered by this mechanism provokes additional activation of the medullary vagal complex, which in turn induces further release of serotonin from the intestinal source. The above mechanism is consistent with the close negative correlations registered between the f-5HT/p-5HT ratio versus heart rate ( $r=-0.56, -0.71, -0.77, P<0.05, P<0.01, P<0.005$  at 60-, 90-, and 120-minute periods, respectively).

In summary, the results from the present study show that the administration of 50 mg of oral L-arginine triggered the inhibition of adrenal sympathetic activity plus the enhancement of the parasympathetic system. The increase of the NA/Ad ratio registered in this oral L-arginine trial fit well with the postulation of a neural over adrenal predominance triggered by the drug. This neural sympathetic predominance avoids neither the

decreased in DBP nor in heart rate reported in this study. The intrinsic neural (DA-mediated) plus the extrinsic parasympathetic (NO-mediated) mechanisms might explain the above ANS changes, respectively. These results afford enough experimental findings that justify the use of L-arginine in the treatment of hypertension syndromes and other cardiovascular disorders.

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