

# Vascular Alterations in High-fat Diet-obese Rats: Role of Endothelial L-arginine/NO Pathway

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## Abstract

**Background:** Mechanisms underlying vascular abnormalities in obesity remain to be completely clarified.

**Objective:** L-arginine/nitric oxide pathway was evaluated on vascular response of high-fat diet-obese rats, focusing on endothelial and smooth muscle cells.

**Methods:** 30-day-old rats were divided in two groups: control (C) and obese (OB, high-fat diet for 30 weeks). After 30 weeks, body weight, adiposity index, blood pressure, and metabolic and endocrine profiles of the animals were recorded. Curves to noradrenaline were obtained in absence and presence of nitric oxide synthase inhibitor (L-NAME,  $3 \times 10^{-4} \text{M}$ ) on intact and denuded thoracic aorta from C and OB rats.

**Results:** Body weight, adiposity index, leptin and insulin levels were increased in OB, while blood pressure was unchanged. Obesity also produced glucose tolerance and insulin resistance. Reactivity to noradrenaline of intact aorta was similar in C and OB rats. L-NAME presence produced a similar increase in maximal responses, but a higher leftward shift of noradrenaline responses in intact aorta from C than in OB rats [EC50 ( $\times 10^{-7} \text{M}$ ): C=1.84 (0.83-4.07), O = 2.49 (1.41-4.38); L-NAME presence C = 0.02 (0.01-0.04)\*, O = 0.21 (0.11-0.40)\*†, \*p < 0.05 vs respective control, †p < 0.05 vs control plus L-NAME, n = 6-7]. None of the protocols altered the reactivity to noradrenaline of denuded aortas.

**Conclusion:** High-fat diet-induced obesity promotes metabolic and vascular alterations. The vascular alteration involved an endothelial L-arginine/NO pathway improvement was probably correlated to diet-induced hyperinsulinemia and hyperleptinemia. The greater resistance to L-NAME effects in aorta of obese rats raises concerns about the lower cardiovascular vulnerability of obese individuals in the presence of associated pathologies that impair NO-system activity. (Arq Bras Cardiol. 2011; [online].ahead print, PP.0-0)

**Keywords:** Obesity; rats; dietary fats; nitric oxide; endothelium, vascular.

## Introduction

Obesity can be defined as a disease in which excess body fat has accumulated so that health may be adversely affected. It is considered to be a global epidemic and constitutes a major public health problem<sup>1,2</sup>. Although the etiology of obesity is complex, several factors have been implicated in its development, especially hypercaloric intake<sup>3</sup>. In this context, a variety of experimental models of obesity exist, however, dietary-induced obesity is the most relevant experimental model regarding human obesity<sup>4</sup>.

Recent investigations have demonstrated that obesity decreases life expectancy and is associated with medical complications, such as type 2 diabetes mellitus, increased

incidence of certain forms of cancer, respiratory complications (obstructive sleep apnea), dyslipidemia, hypertension, atherosclerosis and vascular alterations<sup>1,5</sup>. Current guidelines strongly endorse weight loss for patients who are overweight or obese. This initiative, however, do not distinguish between healthy individuals and those with chronic diseases such as heart failure and atherosclerotic coronary artery disease. Making the distinction between these populations may be important as there is evidence that in patients with chronic diseases, excess weight is paradoxically associated with a decreased risk of adverse outcomes<sup>6-8</sup>.

Several studies have shown that changes in the production and/or release of nitric oxide (NO), a major endothelial factor involved in the regulation of vascular tone<sup>9,10</sup>, are implicated in a variety of pathophysiological responses. Although some controversy exists as to the involvement of NO-pathway on vascular disorders induced by obesity, there is evidence that impairment of NO synthesis represent a central defect triggering many of the vascular abnormalities characteristic of obesity states<sup>11,12</sup>.

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Thus, the aim of the present study was to evaluate the impact of high-fat diet-induced obesity on NO-pathway modulation of arterial tone, focusing on endothelial and smooth muscle cells. This study increases the understanding on the mechanisms of vascular alterations induced by obesity.

## Methods

### Animal

Thirty-day-old male Wistar rats (~150 g) were randomly assigned to one of two groups: control (C) and obese (Ob). The control group was fed a standard rat chow containing 4% fat, 42.7% carbohydrate, and 22% protein; whereas the obese animals received a high-fat diet containing 20% fat, 26.4% carbohydrate, and 20% protein. Each group was fed the diet for 30 weeks (C and Ob;  $n = 22$ ). The high-fat diet was designed in our laboratory and contained powdered commercial Agrocetes™ Animal Chow (Rio Claro, SP, Brazil), industrialized feed, protein supplement, vitamins and minerals. The high-fat diet was calorically rich (high-fat diet = 3.65 kcal/g versus standard diet = 2.95 kcal/g) due to the higher fat composition, made with saturated (20%) and unsaturated fatty acids (80%). All rats were housed in individual cages in an environmentally-controlled clean-air room at  $23 \pm 3^\circ\text{C}$  with a 12-h light/dark cycle and  $60 \pm 5\%$  relative humidity.

All experiments and procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health<sup>13</sup>, and were approved by the Botucatu Medical School Ethics Committee (UNESP, Botucatu, SP, Brazil).

### Nutritional, metabolic and endocrine profiles of the animals

To analyze if dietary-induced obesity was associated with alterations in the nutritional behavior, food consumption was measured daily. Weekly calorie intake was calculated by average weekly food consumption  $\times$  dietary energetic density.

As obesity is defined as an excessive amount of body fat in relation to lean mass<sup>14</sup>, an adiposity index was calculated from the sum of several fat pads. After 12-15 h fasting, animals were anesthetized with sodium pentobarbital (50 mg/kg) and euthanized by decapitation. Animals were thoracotomized and the total body fat (BF) was measured from the sum of the individual fat pad weights: visceral, epididymal and retroperitoneal. The adiposity index was calculated by the ratio: (total body fat/final body weight)  $\times$  100.

Obesity can be accompanied by metabolic and endocrine disturbances<sup>1</sup>, thus glycemic tolerance, leptinemia and insulinemia were analyzed. Related to glycemic tolerance, after fasting for 12-15 h, rats were submitted to a glucose tolerance test (GTT). Blood samples were drawn from the tail at baseline and after administration of glucose (2 g/kg, i.p.)<sup>15,16</sup>. Blood samples were collected at 0, 15, 30, 60, 90 and 120 minutes. Glucose levels were determined using the ACCU-CHEK GO KIT glucose analyzer (Roche Diagnostic Brazil Ltda., Brazil). Glucose tolerance was analyzed from the area under curve of glycemic responses. For biochemical and

hormonal analysis, trunk blood was collected in heparinized tubes, centrifuged at 3,000 g for 15 min at  $4^\circ\text{C}$ . Serum leptin and insulin concentrations were determined by ELISA using commercial kits (Linco Research Inc., USA).

### Homeostasis model assessment (HOMA)

HOMA, an index of insulin resistance, which employs measures of fasting plasma concentrations of glucose and insulin, was calculated according to the previously described method<sup>17</sup>.

### Measurement of systolic blood pressure

Systolic blood pressure was weekly determined by using the non-invasive tail-cuff plethysmographic method (Narco Bio-Systems, Inc., Houston, TX) in conscious rats. The rats were prewarmed at  $35 \pm 2^\circ\text{C}$  for 5 min and placed in a restrainer for blood pressure measurement. The mean of the three stable consecutive measures taken (~1 min apart) was recorded. The cuff pressure was controlled automatically and the systolic pulses detected by the pulse transducer were monitored with the audio signal. Care was taken in selecting an appropriate cuff size for the animal.

### Vascular reactivity

After 30 weeks of high-fat diet or standard rat chow exposure the animals were decapitated. The descending thoracic aorta was excised and trimmed free of adhering fat and connective tissue. Two transverse rings of the same artery, each about 4 mm in length, were cut and mounted at the optimal length for isometric tension recording in organ chambers. One ring served as control, while the endothelium was mechanically removed from the other by gently rubbing the luminal surface<sup>18</sup>. The preparations were mounted in organ baths containing 7 ml of Krebs-Henseleit solution, with composition in mM: NaCl 113.0; KCl 4.7; CaCl<sub>2</sub> 2.5; KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub> 1.1; NaHCO<sub>3</sub> 25.0; Glucose 11.0; ascorbic acid 0.11. The bathing fluid, kept at  $37.0 \pm 0.5^\circ\text{C}$ , was saturated with a gas mixture of 95% O<sub>2</sub>, 5% CO<sub>2</sub>. The preparations were allowed to equilibrate for at least 1 h under a resting tension of 1.5 g, which is optimal in inducing the maximum contraction. Tension was recorded by a physiograph (Ugo Basile).

Cumulative concentration-effect curves were constructed from the response of the tissue to noradrenaline, in the absence and presence of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME  $3 \times 10^{-4}$  M, inhibitor of NO synthase - NOS) (Sigma Chemical Co., St Louis, Missouri, USA). At the end of the curves, single doses of acetylcholine ( $10^{-6}$  M) and sodium nitroprusside ( $10^{-4}$  M) were used to test the integrity of endothelial and smooth muscle layers, respectively.

### Statistical analysis

Blood pressure, HOMA index and nutritional, metabolic and endocrine profiles were expressed as means  $\pm$  standard deviation. Comparisons between groups were performed using the Student *t*-test for independent samples. The mean weekly body weight and the glucose profile of the groups were compared by ANOVA for repeated measures and post hoc Bonferroni-test.

The concentration of vasoactive agents producing a response that was 50% of the maximum (EC50) and maximal responses were calculated in each experiment. The EC50 values, presented as mean with 95% confidence intervals and maximum responses (g of tension), presented as mean  $\pm$  SEM, were compared by ANOVA and post hoc Tukey's test.

The level of significance was considered to be 5%.

## Results

Table 1 shows nutritional, metabolic and endocrine profiles of rats. Obese rats ingested less food than control; however, calorie intake, index of obesity and final body weights at the end of 30 weeks were higher in obese than in the control group. Moreover, blood pressure did not differ between control and obese groups (Table 1). Glucose tolerance was lower in obese rats (Figure 1).

The plasma leptin (C =  $2.2 \pm 1.9$ ; Ob =  $7.8 \pm 3.3^*$  ng/dl, n = 6-7), glucose levels (C =  $107.0 \pm 22.1$ ; Ob =  $126.0 \pm 19.3^*$  mg/dl, n = 6-7), insulin levels (C =  $0.23 \pm 0.08$ ; Ob =  $0.54 \pm 0.07^*$  ng/dl, n = 6-7), HOMA index (C =  $18,319 \pm 3,500$ ; Ob =  $25,151 \pm 4,100^*$ , n = 6-7) were significantly higher in the obese compared to the control group (\*p < 0.05).

In the absence of L-NAME, the reactivity to noradrenaline of intact aorta was shown to be similar between high-fat and standard diet rats (Figure 2 and Table 2). The removal of the endothelium caused a leftward shift of the noradrenaline-aorta curves that was similar in both controls and high-fat diet obese rats (Figure 2 and Table 2). This procedure also determined a similar increase in the maximum response to noradrenaline in aortas from control and obese rats (Figure 1 and Table 2).

The presence of L-NAME produced a leftward shift of the noradrenaline curves that was lower in intact aortas from

high-fat diet obese than control rats (Figure 2 and Table 2). This inhibitor also produced an increase in the aorta maximum response to noradrenaline that was similar between control and obese groups (Figure 2 and Table 2).

Independently of L-NAME presence, the reactivity to noradrenaline did not differ among aortas without endothelium (Figure 2 and Table 2).

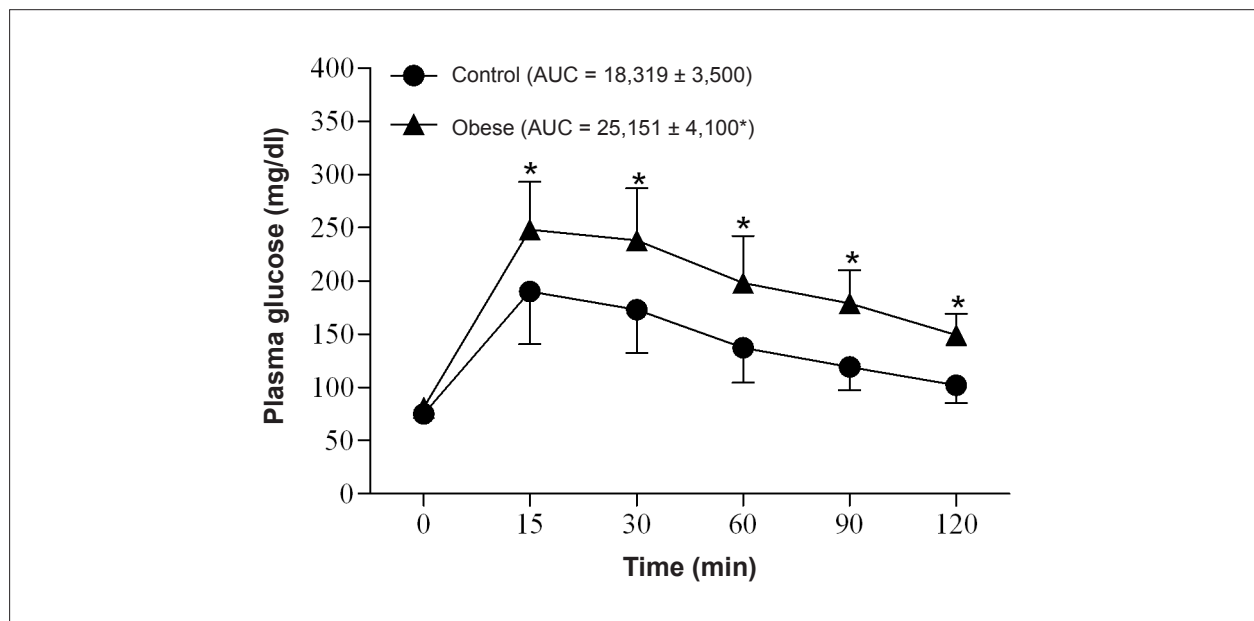
## Discussion

In this study, high-fat diet rats developed obesity characterized by increase in final body weight and fat-pad mass. Although the obese group ingested less food, the higher

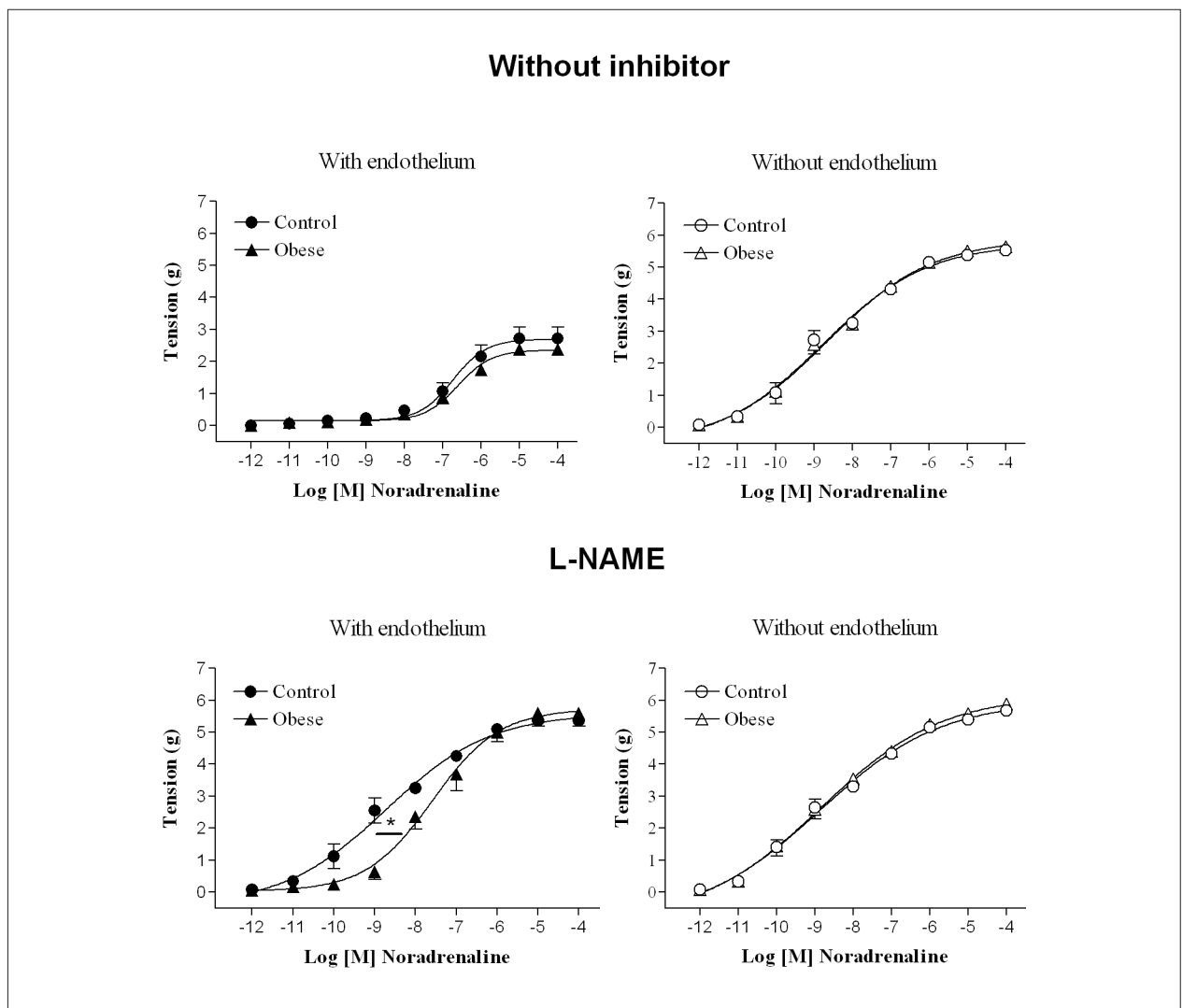
**Table 1 - Effect of high-fat diet-induced obesity on the general characteristics of rats**

Characteristics	Control	Obese
FBW (g)	498 $\pm$ 26	562 $\pm$ 36*
FI (g)	25.1 $\pm$ 1.7	21.8 $\pm$ 1.8*
CI (g kcal/day)	74.3 $\pm$ 5.4	86.1 $\pm$ 6.2*
EF (g)	6.65 $\pm$ 1.96	9.21 $\pm$ 3.80*
RF (g)	7.86 $\pm$ 2.35	11.58 $\pm$ 4.90*
VF (g)	8.31 $\pm$ 3.37	11.43 $\pm$ 4.58*
AI (%)	4.28 $\pm$ 1.65	5.96 $\pm$ 1.54*
FBP (mmHg)	130.7 $\pm$ 5.7	134.6 $\pm$ 6.9

Control rats (n = 6) received a standard diet (4% fat, 42.7% carbohydrate and 22% protein) and obese rats (n = 7) received a high-fat diet (20% fat, 26.4% carbohydrate and 20% protein) for 30 weeks. FBW - final body weight; FI - food intake; CI - calorie intake; EF - epididymal fat; RF - retroperitoneal fat; VF - visceral fat; AI - adiposity index; FBP - final blood pressure. Values are expressed as mean  $\pm$  SD. \*p < 0.05 vs control (Student t-test for independent samples).



**Figure 1 - Glucose tolerance test in control, receiving a standard diet: 4% fat, 42.7% carbohydrate and 22% protein, for 30 weeks (n = 22) and obese, submitted to 30 weeks of high-fat diet: 20% fat, 26.4% carbohydrate and 20% protein (n = 22) rats. Values are expressed as means  $\pm$  standard deviation. AUC - area under the curve for glucose. \*p < 0.05 control vs obese; ANOVA for repeated measures and post hoc Bonferroni test.**



**Figure 2** - Concentration-effect curves to noradrenaline, in the absence and presence of L-NAME ( $3 \times 10^{-4}$  M), obtained in two rings, one with (solid symbol) and the other without (empty symbol) endothelium, of the same thoracic aorta from control (receiving a standard diet: 4% fat, 42.7% carbohydrate and 22% protein, for 30 weeks,  $n = 6$ ) and obese (receiving a high-fat diet: 20% fat, 26.4% carbohydrate and 20% protein, for 30 weeks,  $n = 7$ ) rats. \*  $p < 0.05$ ; ANOVA and Tukey's test.

**Table 2** - Maximal response and EC50 values to noradrenaline obtained in two rings, one with and the other without endothelium, in presence of absence of L-NAME ( $3 \times 10^{-4}$ ), of the same thoracic aorta from control and obese rats

Groups		Maximal response (g)		EC50	
		With endothelium	Without endothelium	With endothelium ( $\times 10^{-7}$ M)	Without endothelium ( $\times 10^{-8}$ M)
Control ( $n = 6$ )	Without inhibitor	2.69 $\pm$ 0.10	5.74 $\pm$ 0.40‡	1.84 (0.83-4.07)	0.16‡ (0.06-0.42)
	L-NAME	4.91 $\pm$ 0.18*	5.60 $\pm$ 0.22	0.02* (0.01-0.04)	0.14 (0.05-0.38)
Obese ( $n = 7$ )	Without inhibitor	2.35 $\pm$ 0.07	5.90 $\pm$ 0.38‡	2.49 (1.41-4.38)	0.22‡ (0.08-0.55)
	L-NAME	5.33 $\pm$ 0.18*	5.72 $\pm$ 0.28	0.21†† (0.11-0.40)	0.15‡ (0.05-0.43)

Control rats received a standard diet (4% fat, 42.7% carbohydrate and 22% protein) and obese rats received a high-fat diet (20% fat, 26.4% carbohydrate and 20% protein) for 30 weeks. Maximal response and EC50 are expressed as mean  $\pm$  SEM and mean followed by 95% confidence interval in parenthesis, respectively. \* $p < 0.05$  vs respective control; † $p < 0.05$  vs control/L-NAME; ‡ $p < 0.05$  vs respective aorta with endothelium; ANOVA and Tukey's test.  $n$  - number of animals.

weight gain exhibited by these animals was most likely due to the increased calorie intake and feed efficiency in relation to controls. In addition, the obese rats developed metabolic disorders such as glucose intolerance, hyperinsulinemia and hyperleptinemia, characteristics commonly related to human obesity<sup>15,19,20</sup>. Insulin-resistance, defined as decreased sensitivity and/or responsiveness to metabolic actions of insulin, is a cardinal feature of diabetes, obesity and dyslipidemia<sup>21</sup>. In this context, HOMA index was shown to be increased in obese rats indicating insulin-resistance development in these animals as previously reported by Naderalli et al<sup>22</sup> in diet-fed rats.

A number of experimental studies have reported a detrimental outcome of excessive high-energy diet on vascular reactivity. For example, high-fat diet impairs endothelium-dependent vasorelaxation<sup>23,24</sup>. This may, at least in part, explain the increased risk of vascular disease in obese subject and animals. Moreover, antioxidant therapy improves endothelial function<sup>25,26</sup>, suggesting a diet-induced oxidative stress in vasculature. In the present study, diet-fed obese rats had significant vascular alteration, particularly a marked endothelial improvement that was unveiled in the presence of L-NAME. In other words, diet-induced obesity did not alter the aorta reactivity to noradrenaline in the absence of L-NAME. However, the presence of this inhibitor produced a leftward shift of noradrenaline curves that was lower in the aorta of obese in relation to control rats. Moreover, the high-fat diet did not induce any changes in the aorta smooth muscle reactivity to noradrenaline. This conclusion could be inferred from the observation that, regardless of L-NAME presence, the reactivity of denuded aortas did not differ between control and obese rats.

As the aorta from diet-fed obese rats was more resistant to the effects of L-NAME, it can be hypothesized that the endothelial L-arginine/NO pathway is improved in the vasculature of obese rats. Moreover, this increased NO bioavailability in high-fat diet rats could represent an adaptive mechanism to counteract the reported detrimental outcome of excessive high-energy diet on vascular reactivity in obesity, mainly oxidative stress<sup>25,26</sup>. The improvement in endothelial NO pathway could also explain the absence of changes in blood pressure of obese rats, a parameter often reported to

be increased in human and animal obesity<sup>27,28</sup>. These findings confirm the "obesity paradox"<sup>6</sup> and could help to understand the mechanisms responsible for this condition.

Finally, the literature also reported that vasodilation leading to increased blood flow is a major physiological consequence of insulin-stimulated production of NO in vascular endothelium<sup>29</sup>. Moreover, leptin hormone is able to directly activate NO production in vessels and this activation is dependent on endothelium integrity<sup>30</sup>. Thus, the hyperinsulinemia and hyperleptinemia observed in high-fat diet animals could be partially responsible for the increased endothelial NO production and/or release in the vasculature of obese rats.

Overall, this study indicates that high-fat diet-induced obesity promotes metabolic and vascular alterations. The vascular alteration was characterized by an endothelial L-arginine/NO pathway improvement, probably correlated to diet-induced hyperinsulinemia and hyperleptinemia. Finally, the greater resistance to L-NAME effects in the aorta of obese rats in relation to controls raises concerns about the lower cardiovascular vulnerability of obese individuals in the presence of associated pathologies that impair NO-system activity.

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## Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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## Study Association

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