Systemic But Not Central Nervous System Nitric Oxide Synthase Inhibition Exacerbates the Hypertensive Effects of Chronic Melanocortin-3/4 Receptor Activation

Jussara M. do Carmo, Mirian Bassi, Alexandre A. da Silva, John E. Hall

Abstract—We examined whether systemic or central nervous system (CNS) inhibition of nitric oxide synthase exacerbates the cardiovascular responses of chronic CNS melanocortin 3/4 receptor activation. Sprague-Dawley rats implanted with telemetry probes, venous catheters, and intracerebroventricular (ICV) cannulae were divided in 3 groups. After control measurements, the NO synthase inhibitor L-NAME was infused (10 μg/kg/min intravenous) for 17 days and, starting on day 7 of L-NAME infusion, the melanocortin 3/4 receptor agonist melanotan II (MTII; 10 ng/hr; group 1) or saline vehicle (group 2) was infused ICV for 10 days. A third group not treated with L-NAME also received MTII ICV. Melanocortin 3/4 receptor activation caused a greater increase in mean arterial pressure (MAP) and heart rate in rats treated with intravenous L-NAME (35±6 mm Hg and 56±8 bpm) than L-NAME plus vehicle or MTII alone (22±5 and 9±2 mm Hg, and 26±14 and 27±5 bpm), despite a 58% and 50% reduction in food intake during the first 6 days of MTII infusion. To test if the amplified pressor response to MTII after L-NAME was attributable to a reduction in nitric oxide availability in the brain, we also infused L-NAME directly into the CNS alone or in combination with MTII. ICV infusion of L-NAME plus MTII caused only ≈10 mm Hg increase in MAP with no change in heart rate, similar to the effects of ICV infusion of MTII alone, whereas ICV infusion of L-NAME alone had no effect on MAP. These results suggest that reduction in peripheral, but not CNS, nitric oxide production augments MAP sensitivity to CNS melanocortin 3/4 receptor activation. (Hypertension. 2011;57:428-434.)

Key Words: blood pressure ■ central nervous system ■ food intake ■ melanocortin system

One of the most important regulators of energy balance and body weight homeostasis is the central nervous system (CNS) melanocortin system. Activation of pro-opiomelanocortin neurons leads to production and release of α-melanocyte-stimulating hormone, which, in turn, activates melanocortin 3/4 receptors (MC3/4R), suppresses appetite, and increases energy expenditure by raising sympathetic nerve activity to thermogenic tissues, such as the brown adipose tissue.1–3 Dysfunction of the melanocortin system, either by mutations of the MC4R or by pro-opiomelanocortin deficiency, in humans and rodents is associated with marked hyperphagia and reduced energy expenditure, leading to severe early-onset obesity that is accompanied by many characteristics of the metabolic syndrome, including hyperglycemia, insulin resistance, and hyperleptinemia.4–6 Some studies suggest that a defective melanocortin system may account for as much as 5% to 6% of early-onset morbid obesity in humans.7–9

In addition to its role in regulating appetite and energy balance, acute and chronic MC3/4R activation stimulates sympathetic nerve activity to tissues that regulate cardiovascular function, including the heart, blood vessels, and kidneys, causing increased blood pressure (BP) and heart rate (HR).10–12 Studies in experimental animals and in humans suggest that a functional MC3/4R may be necessary for obesity to cause hypertension. For example, BP of MC4R-deficient mice is not elevated despite severe obesity, insulin resistance, hyperinsulinemia, and other features of the metabolic syndrome.13,14 Likewise, humans with dysfunctional MC4R exhibit severe obesity and metabolic syndrome but are not hypertensive and actually have lower BP than control subjects.15 These observations support the concept that MC3/4R activation is required for excess weight gain to increase BP. Although previous studies have shown that chronic MC3/4R activation in lean animals causes only modest increases in BP of ≈7 to 10 mm Hg, it is possible that other factors associated with obesity may exacerbate the pressor actions of CNS melanocortin system activation. One potential factor is reduced nitric oxide (NO) availability that may occur after the development of endothelial dysfunction associated with prolonged obesity.16,17 To the extent that obesity impairs NO availability, one might expect greater BP responses to activation of the CNS melanocortin system. The present study...
therefore was designed to test this hypothesis by activating the CNS melanocortin system with chronic intracerebroventricular (ICV) infusion of the specific MC3/4R agonist, melanotan II (MTII), in rats treated with an inhibitor of NO synthesis, L-NAME.

Some studies also suggest that obesity reduces NO formation in the brain, a change that also could amplify the chronic BP effects of CNS melanocortin activation in obesity. Therefore, we also tested whether ICV infusion of L-NAME to inhibit CNS NO formation would exacerbate the hypertensive effects of chronic MC3/4R activation. Our results indicate that systemic, but not ICV, administration of L-NAME markedly enhanced the hypertension and tachycardia associated with chronic MC3/4R activation.

Materials and Methods

All experimental procedures conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

Animal Surgery
Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with sodium pentobarbital (50 mg/kg), and atropine sulfate (0.37 mg/kg) was administered to prevent excessive airways secretion. A telemetric pressure transmitter (Model TA111PAC40; Data Sciences International) was implanted into the abdominal aorta distal to the kidneys under sterile conditions as previously described. A femoral vein catheter was also implanted and the tip was advanced into the inferior vena cava. The catheter was exteriorized through a stainless steel button that was implanted subcutaneously in the scapular region. A stainless steel cannula (26-gauge, 10-mm-long) was implanted into the brain left lateral ventricle using coordinates previously described. Ten days after recovery from surgery, accuracy of the cannula was tested by measuring the dipsogetic response to an acute injection of 100 ng of angiotensin.

After recovery from surgery, each rat was individually housed in a metabolic cage. The venous catheter was connected to a swivel and a continuous infusion of saline was maintained throughout the study. All rats received water and food ad libitum and total daily sodium intake was maintained constant at ~3.1 mEq/d by continuous infusion of 20 mL/d of 0.9% saline combined with a sodium-deficient rat chow (0.006 mmol sodium/gram of food; Harlan Teklad).

Experiment 1: Responses to Chronic MC3/4R Activation During Systemic NO Synthase Inhibition

Two groups of rats were used in these experiments. After a 5-day control period, rats received intravenous (IV) L-NAME (10 µg/kg/min) infusion for 17 consecutive days. During the last 10 days of L-NAME infusion, the rats received either an IV infusion of the vehicle (0.9% saline, 0.5 µL/h, n = 6) or the MC3/4R agonist, MTII (10 ng/h, n = 6), via an osmotic minipump implanted subcutaneously in the scapular region and connected to the ICV cannula via tygon tubing. The 10-day experimental period was followed by 5 days of recovery, during which all drug infusions were terminated.

Experiment 2: Responses to Chronic MC3/4R Activation During CNS NO Synthase Inhibition

Four groups of rats were used in these experiments. After a 5-day control period, the rats received an ICV infusion of either the vehicle (0.9% saline, 0.5 µL/h, n = 6, group 1), the MC3/4R agonist MTII alone (10 ng/h, n = 6, group 2), L-NAME alone (150 µg/kg/d, group 3), or MTII plus L-NAME combined for 10 days using osmotic minipumps (group 4). The experimental period was followed by a 5-day recovery period.

In experiments 1 and 2, food intake, mean arterial pressure (MAP), and HR were recorded 24 hours per day and daily averages were determined. Blood samples were collected after 6 hours of fasting during control, experimental, and recovery periods for measurement of plasma glucose, insulin, and leptin levels.

Analytic Methods

Plasma leptin and insulin concentrations were measured using enzyme-linked immunosorbent assay (R&D Systems and Crystal Chem, respectively), and plasma glucose concentrations were determined using the glucose oxidation method (Beckman glucose analyzer 2; Beckman).

Statistical Methods

The results are expressed as means±SEM. The data were analyzed by paired t test or 1-way ANOVA with repeated measures, followed by Dunnett post hoc test for comparisons between control and experimental values within each group when appropriate. Comparisons between different groups were made by 2-way ANOVA followed by Bonferroni post hoc test when appropriate. Statistical significance was accepted at a level of P<0.05.

Results

Plasma Glucose, Insulin, and Leptin Responses to MC3/4R Activation and IV or ICV L-NAME Infusion

As shown in the Table, chronic central MC3/4R activation with MTII caused significant reductions in plasma concentrations of glucose (132±2 to 120±2 mg/dL) and insulin (22.0±3.0 to 10.1±2.1 µU/mL). Systemic NOS inhibition with IV L-NAME infusion did not alter the effects of MC3/4R activation to reduce plasma glucose (127±10 to 90±12 mg/dL) and insulin levels (19.4±2.0 to 12.0±3.2 µU/mL). Intravenous or ICV infusions of L-NAME alone did not significantly alter plasma glucose or insulin levels. Combined ICV administration of MTII plus L-NAME also did not alter plasma glucose and insulin levels. No significant changes in plasma leptin levels were observed in any of the groups.

Food and Water Intake and Body Weight Responses to Chronic MC3/4R Activation and IV or ICV L-NAME Infusion

Chronic ICV MC3/4R agonist infusion alone or in combination with systemic NOS inhibition significantly reduced food intake during the first 6 days, after which food intake returned to control values (Figure 1A, B). Systemic NOS inhibition alone slightly reduced food intake (Figure 1A). ICV L-NAME infusion did not alter the anorectic effect of MC3/4R agonist infusion (Figure 2A, B). ICV L-NAME administration alone caused only a small reduction in food intake during the first 3 days of treatment (Figure 2A, B).

Chronic ICV MC3/4R agonist infusion alone or combined with systemic or CNS NOS inhibition significantly reduced body weight (Table). No significant changes in body weight were observed in the vehicle group or when L-NAME was infused alone (Table).

Chronic ICV infusion of MTI alone or in combination with ICV L-NAME infusion caused no significant changes in water intake (control, 25±2 mL; MTII ICV, 29±3 mL;
MAP and HR Responses to Chronic MC3/4R Activation and Central L-NAME Infusion

To test whether the exacerbated pressor responses to CNS MC3/4R activation in rats receiving intravenous L-NAME infusion were caused by reduced NO availability in the CNS, we investigated the chronic MAP and HR responses to MTII in rats that received L-NAME infusion directly into the brain ventricle. ICV infusion of L-NAME, however, did not augment the MAP or HR responses to MTII (average increase of 8 mm Hg during the last 5 days of infusion; Figure 5A, B). In fact, blockade of NOS in the CNS attenuated the increase in HR during chronic MTII infusion (Figure 5C). Central infusion of L-NAME alone had no effect on MAP or HR (Figure 5).

### Discussion

In the present study we demonstrated that chronic CNS MC3/4R activation increased MAP and HR despite a 60% reduction in food intake and associated weight loss. We also observed that the MAP and HR responses to MC3/4R activation were exacerbated by systemic but not CNS inhibition of NOS using L-NAME. In addition, we showed that chronic MC3/4R activation reduced plasma glucose and insulin levels, and these effects were attenuated by central L-NAME administration.

### Metabolic and Hormonal Effects of Chronic MC3/4R Activation and Inhibition of NO Synthesis

As reported in previous studies, chronic central activation of MC3/4R with MTII transiently reduced food intake by ~60% and promoted weight loss. Although the precise mechanisms accounting for the return of food intake to control values during prolonged MC3/4R activation are not fully understood, evidence suggests that activation of orexigenic peptides, including neuropeptide Y or agouti-related peptide, may play a role. Because neuropeptide Y neurons in the hypothalamic area make synaptic contact with NO neurons that express Y1 receptors, and because stimulation of Y1 receptors enhances NO production in the hypothalamus and cerebral cortex in rats, we expected that L-NAME infusion might exacerbate the anorexigenic effect of MC3/4R.
Figure 1. A, Response to chronic intravenous (IV) L-NAME infusion (10 µg/kg/min) plus intracerebroventricular (ICV) infusion of vehicle or L-NAME plus ICV infusion of the melanocortin 3/4 receptor (MC3/4R) agonist, melanotan II (MTII; 10 ng/hr), on food intake. B, Percent change in food intake in response to IV L-NAME infusion, L-NAME plus ICV MTII infusion, or MTII alone in Sprague-Dawley rats. Data are presented as mean±SEM. *P<0.05 vs control period (1-way ANOVA repeated-measures). Comparisons among groups using 2-way ANOVA with repeated measures indicated a group effect for L-NAME IV plus MTII ICV and vehicle plus MTII ICV vs L-NAME IV plus vehicle ICV.

Figure 2. A, Response to chronic intracerebroventricular (ICV) L-NAME infusion (150 µg/kg/d), L-NAME plus melanocortin 3/4 receptor (MC3/4R) agonist, melanotan II (MTII; 10 ng/hr), or MTII alone on food intake. B, Percent change in food intake in response to chronic ICV L-NAME infusion, L-NAME plus MTII (10 ng/hr), or MTII alone in Sprague-Dawley rats. Data are presented as mean±SEM. *P<0.05 vs control period (1-way ANOVA repeated-measures). Comparisons among groups using 2-way ANOVA with repeated measures indicated a group effect for L-NAME plus MTII ICV and MTII ICV alone vs L-NAME ICV group.

Activation. Although chronic systemic inhibition of NOS with IV L-NAME infusion caused a small reduction in food intake, it did not influence the anorexigenic action of MC3/4R activation with MTII. When administered directly in the cerebral ventricle, L-NAME also failed to enhance the appetite suppressant effect of MC3/4R activation. These observations suggest that the ability of MC3/4R activation to suppress food intake does not depend on NO levels in the CNS and that generalized reduction of NO availability in the CNS does not have a major effect on appetite regulation. The causes of the small reduction in appetite during IV L-NAME administration are unclear but may be related to the multiple systemic effects, including the marked cardiovascular actions, of blocking NO synthesis.

MC3/4R activation also reduced plasma glucose and insulin levels in parallel with the reductions in food intake and body weight. Previous studies demonstrated that activation of the MC3/4R is accompanied by increased insulin sensitivity and peripheral glucose utilization. We also have shown that the ability of MTII to reduce insulin levels while at the same time reducing plasma glucose levels is well-maintained in a dietary model of obesity. In the present study, we observed no alterations in the ability of MTII to reduce plasma insulin and glucose levels when L-NAME was infused systemically; however, surprisingly, we found that this effect was abolished when L-NAME was infused into the CNS. Therefore, it is possible that despite not being an important component of the appetite suppressing actions of MC3/4R activation, increased NO levels in response to stimulation of MC3/4R may contribute, at least in part, to the effects of MTII on insulin and glucose regulation. However, additional studies are needed to test this possibility because this the present study was not designed to examine the mechanism by which central inhibition of NOS alters insulin sensitivity and enhances glucose utilization during chronic MC3/4R activation.
Hemodynamic Effects of MC3/4R Activation During Inhibition of NO Synthesis

The present study and previous studies demonstrated that MC3/4R activation caused significant and sustained increases in arterial pressure and HR, despite reductions in food intake and body weight that would tend to lower arterial pressure and HR. Moreover, the effects appear to be attributable to increased adrenergic activation because they were abolished by combined α-adrenergic and β-adrenergic receptor blockade. The hypertensive effects of chronic MC3/4R activation, however, are modest in normal animals. The major goal of the present study was to determine whether impaired synthesis of NO, which often occurs in obese subjects with endothelial dysfunction, exacerbates the hypertensive effects of MC3/4R activation. To our knowledge, previous studies have not examined the role of NO in modulating the chronic cardiovascular actions of MC3/4R activation, although several studies suggest that obesity is often associated with endothelial dysfunction and reduced vascular NO bioavailability in experimental animals and in humans.

Our current study demonstrated that a reduction in systemic NO availability exacerbates the hypertension and tachycardia responses to chronic MC3/4R activation despite concomitant marked reductions in food intake and body weight. These findings are consistent with the hypothesis that reduced systemic NO availability and endothelial dysfunction in obese subjects may predispose them to enhanced cardiovascular responses to CNS melanocortin activation and contribute to the development of obesity-induced hypertension. However, the degree of inhibition of NO synthesis achieved in our studies with L-NAME administration may be greater than that found in many obese subjects, especially if severe hypertension and atherosclerosis have not yet developed.

Figure 3. A, Response to chronic intravenous (IV) L-NAME infusion (10 µg/kg/min) plus intracerebroventricular (ICV) infusion of vehicle or L-NAME plus ICV infusion of the melanocortin 3/4 receptor (MC3/4R) agonist, melanotan II (MTII; 10 ng/hr), on mean arterial pressure (MAP). B, Change in MAP during IV L-NAME infusion, L-NAME plus ICV MTII infusion, or MTII alone in Sprague-Dawley rats. Data are presented as mean±SEM. *P<0.05 vs control period (1-way ANOVA repeated-measures). Comparisons among groups using 2-way ANOVA with repeated measures indicated a group effect for L-NAME IV plus MTII ICV vs MTII ICV alone and L-NAME IV plus vehicle ICV groups.

Figure 4. A, Response to chronic intravenous (IV) L-NAME infusion (10 µg/kg/min) plus intracerebroventricular (ICV) infusion of vehicle or L-NAME plus ICV infusion of the melanocortin 3/4 receptor (MC3/4R) agonist, melanotan II (MTII; 10 ng/hr), on heart rate (HR). B, Change in HR during IV L-NAME infusion, L-NAME plus ICV MTII infusion, or MTII alone in Sprague-Dawley rats. Data are presented as mean±SEM. *P<0.05 vs control period (1-way ANOVA repeated-measures). Comparisons among groups using 2-way ANOVA with repeated measures indicated a group effect for L-NAME IV plus MTII ICV vs MTII ICV alone and L-NAME IV plus vehicle ICV groups.
Figure 5. A, Response to chronic intracerebroventricular (ICV) L-NAME infusion (150 μg/kg/d), L-NAME plus melanocortin 3/4 receptor (MC3/4R) agonist, melanotan II (MTII; 10 ng/hr), or MTII alone on mean arterial pressure (MAP). B, Change in MAP during chronic ICV L-NAME infusion, L-NAME plus MTII, or MTII alone in Sprague-Dawley rats. Data are presented as mean±SEM. *P<0.05 vs control period (1-way ANOVA repeated-measures). Comparisons among groups using 2-way ANOVA with repeated measures indicated a group effect for MTII ICV alone vs L-NAME plus MTII ICV and L-NAME ICV groups.

There is also evidence that obesity may reduce NOS activity in the hypothalamus,18,30,31 although the functional significance of these changes and their potential interactions with the CNS melanocortin system in regulating BP are still unclear. We therefore hypothesized that the exaggerated BP and HR responses to chronic MC3/4R activation during systemic NOS inhibition were mediated, at least in part, by a reduction in NO levels in the CNS that could enhance the ability to MTII to trigger sympathetic activation. Previous studies have suggested that NO plays an important role in regulating CNS activity, and that central L-NAME administration increases CNS activity and raises BP.25 Also, because L-NAME crosses the blood–brain barrier, it is possible that some of the effects of systemically administered L-NAME could have been mediated though CNS actions. However, contrary to what we anticipated, central infusion of L-NAME did not alter BP and HR in normotensive rats and also failed to exacerbate the cardiovascular responses to chronic MC3/4R activation.

One potential explanation for the lack of a BP or HR response to central L-NAME administration in the present study compared to the study by Qadri et al32 may be that we measured BP and HR 24 hours per a day using telemetry, whereas in the previous study BP and HR were measured for only 90 minutes via a catheter implanted in the femoral artery 24 hours before the measurement.32 We are confident that we achieved at least a comparable degree of NOS inhibition because we used a dose 3-times greater than that in the study by Qadri et al.32 Taken together, these findings indicate that reduced systemic NO availability markedly enhances the BP and HR responses to chronic central activation of the MC3/4R, and that these exacerbated responses are unlikely to be mediated by inhibition of CNS NO levels synthesis. It is also possible, however, that chronic ICV infusion of L-NAME may lead to widespread reductions in NO in the brain, whereas peripheral L-NAME administration may reduce NO levels in certain key cardiovascular centers in the brain that are exposed to factors present in the systemic circulation. However, additional studies are necessary to test this possibility.

Perspectives
The CNS melanocortin system appears to play an important role in linking obesity with sympathetic activation and hypertension. The fact that obesity is also associated with development of endothelial dysfunction and impaired NO production and/or availability suggests that the degree of sympathetic activation and consequent increase in arterial pressure and heart rate evoked by MC3/4R activation in obese individuals may be importantly modulated by various factors, including endothelial dysfunction and sensitivity of the neurons to factors that activate the pro-opiomelanocortin–MC3/4R pathway. We did not determine the precise mechanisms by which reduced NO availability may enhance the effects of MC3/4R activation in the regulation of BP and HR. Although our previous studies suggest that CNS MC3/4R activation increases BP mainly by stimulation of sympathetic activity, it is still unclear how the peripheral effects of inhibiting NO synthesis in the blood vessels, heart, and kidneys interact to augment the hypertensive actions of central MC3/4R activation. Unraveling the mechanisms by which these factors potentiate the hypertensive responses of CNS melanocortin activation will significantly improve our understanding of obesity-induced hypertension.
Acknowledgments
The authors thank Haiyan Zhang for the insulin and leptin assays, and Benjamin Pace, Calvin Torrey, and John Rushing for technical support.

Sources of Funding
This research was supported by the National Heart, Lung, and Blood Institute grant PO1HL-51971 (J.E.H.) and by an American Heart Association Scientist Development grant (A.A.S.).

Disclosure
None.

References
Systemic But Not Central Nervous System Nitric Oxide Synthase Inhibition Exacerbates the Hypertensive Effects of Chronic Melanocortin-3/4 Receptor Activation

Jussara M. do Carmo, Mirian Bassi, Alexandre A. da Silva and John E. Hall

Hypertension. 2011;57:428-434; originally published online January 24, 2011;
doi: 10.1161/HYPERTENSIONAHA.110.163931

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/57/3/428