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Obesity induced by neonatal treatment with monosodium glutamate impairs microvascular reactivity in adult rats: Role of NO and prostanoids

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Abstract *Background and aim:* given that obesity is an independent risk factor for the development of cardiovascular diseases we decided to investigate the mechanisms involved in microvascular dysfunction using a monosodium glutamate (MSG)-induced model of obesity, which allows us to work on both normotensive and normoglycemic conditions.
Methods and results: Male offspring of Wistar rats received MSG from the second to the sixth day after birth. Sixteen-week-old MSG rats displayed higher Lee index, fat accumulation, dyslipidemia and insulin resistance, with no alteration in glycemia and blood pressure. The effect of norepinephrine (NE), which was increased in MSG rats, was potentiated by L-nitro arginine methyl ester (L-NAME) or tetraethylammonium (TEA) and was reversed by indomethacin and NS-398. Sensitivity to acetylcholine (ACh), which was reduced in MSG rats, was further impaired by L-NAME or TEA, and was corrected by indomethacin, NS-398 and tetrahydrobiopterin (BH4). MSG rats displayed increased endothelium-independent relaxation to sodium nitroprusside. A reduced prostacyclin/tromboxane ratio was found in the mesenteric beds of MSG rats. Mesenteric arterioles of MSG rats also displayed reduced nitric oxide (NO) production along with increased reactive oxygen species (ROS) generation; these were corrected by BH4 and either L-NAME or superoxide dismutase, respectively. The protein expression of eNOS and cyclooxygenase (COX)-2 was increased in mesenteric arterioles from MSG rats.
Conclusion: Obesity/insulin resistance has a detrimental impact on vascular function. Reduced NO bioavailability and increased ROS generation from uncoupled eNOS and imbalanced release of COX products from COX-2 play a critical role in the development of these vascular alterations

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Introduction

The prevalence of obesity is rising to epidemic proportions not only in industrialized nations but also in developing nations [1,2]. Obesity is an independent risk factor for hypertension, coronary artery disease, stroke, and type 2 diabetes [3]. Endothelial dysfunction, which presents as an altered ability of the endothelium to maintain vascular homeostasis through the release of endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs), is present in obesity and may be crucial for the increased cardiovascular risk associated with this condition [4,5].

The monosodium glutamate (MSG)-induced obese rat is a model associated with insulin resistance and dyslipidemia that may occur without the presence of hypertension or type 2 diabetes, depending on the age at which the animals are studied [6–8]. The administration of MSG to newborn rats results in distinctive lesions in hypothalamic arcuate nucleus (ARC) neurons. The neuronal loss impairs insulin and leptin signaling and impacts energy balance as well as pituitary and adrenal activity. In contrast to other models of obesity, MSG-treated rats are characterized by increased plasma levels of corticosterone as well as increased lipogenesis and reduced lipolysis in the adipose tissue, despite their normophagia [9–13].

An understanding of the alterations associated with MSG-induced obesity is of great relevance because the ARC is among the principal sites that regulate energy homeostasis [14]. Although the endocrine, metabolic, and autonomic aspects of MSG-induced obesity have been extensively studied, the association between MSG and the development of vascular alterations is less understood. Therefore, we decided to investigate the mechanisms involved in alterations of vascular reactivity using this obesity model, which allows evaluation under normotensive and normoglycemic conditions [6–8]. The role of nitric oxide (NO), reactive oxygen species (ROS), endothelium-derived hyperpolarizing factor (EDHF), and cyclooxygenase (COX) pathways were investigated.

Methods

Animals

The investigation was approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, University of Sao Paulo (Protocol n°007/04) and conforms to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Male Wistar rats received subcutaneous injections of MSG [4.0 g/kg body weight] dissolved in 0.9% NaCl or an equivalent volume of vehicle from the second to the sixth day after birth. Breeding conditions were followed as previously described [15].

At 16 weeks of age, the rats were weighed and placed in metabolic cages to measure water and food consumption. Blood samples were taken from the descending aorta after food deprivation (5 h) under sodium thiopental anesthesia (50 mg/kg, Cristália, Brazil). Glucose levels and lipid profiles were assessed spectrophotometrically (Celm, Brazil). Insulin levels were determined by radioimmunoassay (Linco, USA). The Homeostasis Model Assessment (HOMA-IR), an index of insulin resistance, was calculated using this equation: fasting insulin (μIU/mL) × fasting glucose (mmol/L)/22.5 [16]. Lee's obesity index was calculated as follows: body weight$^{1/3}$(g)/nasal–anal length(cm) × 100. White adipose tissue and lean mass were weighed.

The blood pressure (BP) was measured in unanesthetized animals by an indirect tail-cuff method (PowerLab 4/S, ADInstruments, Australia).

Intravenous insulin tolerance test

Tail blood samples were collected before and 4, 8, 12 and 16 min after an intravenous injection of regular insulin (0.75 U/kg b.w., Biobras, Brazil). The constant rate for blood glucose disappearance during the test (*k*ITT) was calculated based on the linear regression of the neperian logarithm of glucose concentrations.

Vascular reactivity in the perfused mesenteric arteriolar bed

The perfused mesenteric arteriolar bed (MAB) was prepared as previously described [17]. Under anesthesia, the abdominal cavity was opened and a polyethylene cannula was inserted into the superior mesenteric artery. The whole MAB was cut close to the intestinal border, transferred to

Table 1 Anthropometric and several metabolic characteristics in sixteen-week-old control and MSG-damaged Wistar rats.

Parameter	Control	MSG
Body weight (g)	386.6 ± 8.3	333.8 ± 7.8[a]
Lee index, (×100)	29.05 ± 0.22	30.43 ± 0.24[a]
Retroperitonial WAT, (g/100 g)	0.97 ± 0.07	2.81 ± 0.11[a]
Periepididymal WAT, (g/100 g)	1.16 ± 0.10	2.62 ± 0.15[a]
Soleus muscle, (g/100 g)	0.033 ± 0.003	0.036 ± 0.002
Extensor digitorum longus muscle, (g/100 g)	0.030 ± 0.002	0.032 ± 0.001
Food intake (g/100 g/day)	5.16 ± 0.31	5.24 ± 0.35
Water intake (mL/100 g/day)	8.3 ± 0.1	8.1 ± 0.2
Total Colesterol, (mg/dL)	71.6 ± 4.3	64.4 ± 3.2
Triacylglycerols, (mg/dL)	48.8 ± 11.6	123.8 ± 18.3[a]
LDL-cholesterol, (mg/dL)	24.3 ± 3.7	38.2 ± 5.8[a]
Glucose, (mg/dL)	116.0 ± 3.8	115.8 ± 3.0
Insulin, (ng/mL)	2.1 ± 0.33	3.5 ± 0.34[a]
*k*ITT, (%/min)	4.2 ± 0.20	2.8 ± 0.30[a]
HOMA-IR index	13.8 ± 2.3	25.5 ± 2.8[a]
Blood pressure, (mmHg)	113.2 ± 1.5	108.0 ± 2.7

Values are mean ± SEM; WAT, white adipose tissue; LDL, low density lipoprotein; *k*ITT, constant rate for blood glucose disappearance; HOMA-IR, homeostasis model assessment-insulin resistance.
[a] $P < 0.05$ vs. control. $N = 10–12$/group.

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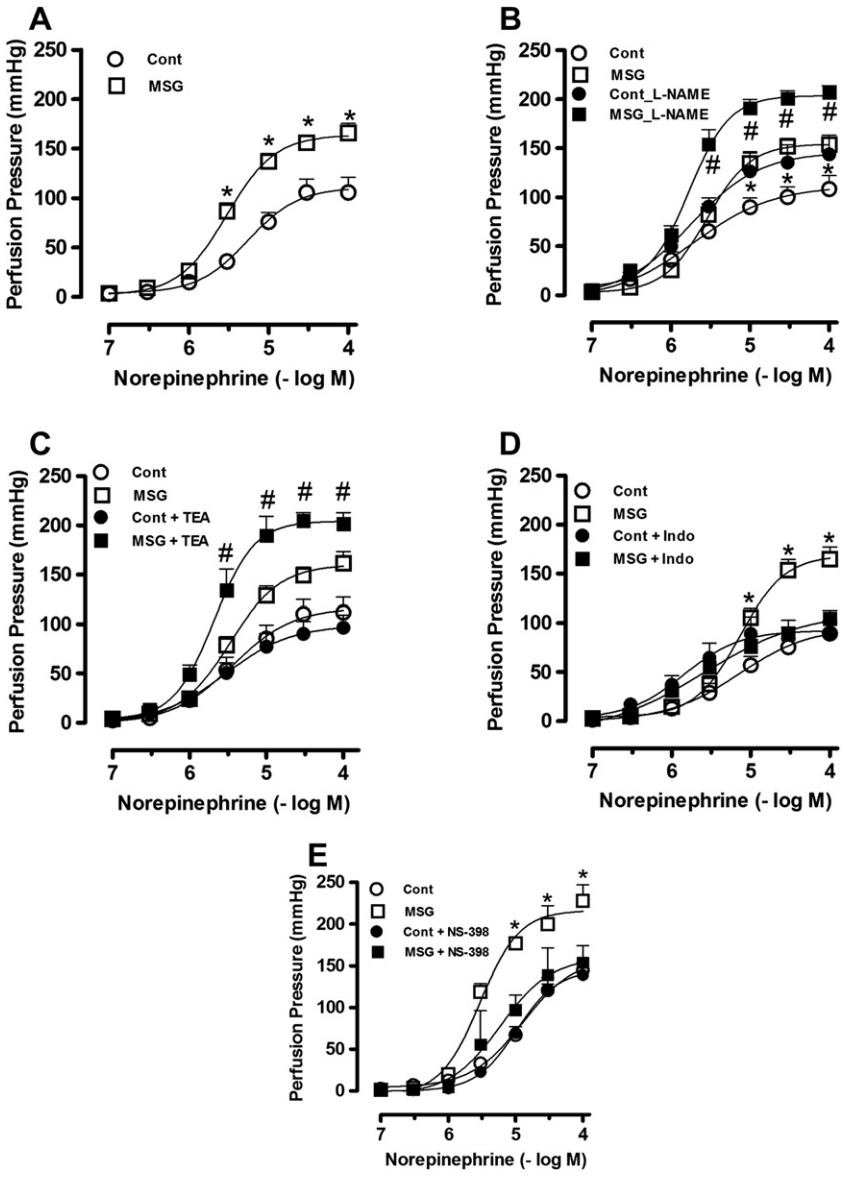

Figure 1 Concentration-response curves to norepinephrine in mesenteric arteriolar beds from control (Cont) and monosodium glutamate (MSG)-treated rats in the absence (A) or presence of L-NAME (B, 100 μM), TEA (C, 2 mM), indomethacin (D, Indo 10 μM) or NS-398 (E, 1 μM). Data are expressed as mean ± SEM of perfusion pressure increase. $n = 7–10$/group. *$P < 0.05$ vs. control. #$P < 0.05$ vs. MSG.

an organ bath at 37 °C and perfused at a constant flow rate (2 mL/min) by a peristaltic pump with Krebs-Henseleit solution (pH 7.4) containing 5% CO_2 and 95% O_2. Vascular responses were evaluated as changes in the perfusion pressure, which was measured with a pressure transducer (BP Transducer, ADInstruments, Australia) and recorded using a digital acquisition system (Power Lab, ADInstruments).

Concentration-response curves for norepinephrine (NE, 0.1–100 μM), potassium chloride (KCl, 5–225 mM), acetylcholine (ACh, 0.001–30 μM), and sodium nitroprusside (SNP, 0.001–10 μM) were obtained. All curves were determined in the presence of desipramine (inhibitor of NE uptake, 10 nM). The vasodilator responses were determined in NE-contracted preparations at a concentration that produced 80% of the maximal contractile response.

To determine the role of NO, prostanoids and EDHF on the NE and ACh responses, L-nitro arginine methyl ester (L-NAME, 100 μM), a NO synthase inhibitor, tetrahydrobiopterin (BH_4, 1 μM), a cofactor for NOS, indomethacin (10 μM), a COX inhibitor, NS-398 (1 μM), a selective COX-2 inhibitor, or tetraethylammonium (TEA, 2 mM), a K^+-channel blocker, were used. The concentrations of these agents used in our experiments were based on previous data [18].

Prostanoid release measurements

TXA_2 and PGI_2 (estimated from measurements of 11-dehydro-TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively) were assessed in 1-mL samples of perfusate collected before and after stimulation with NE (100 nM) or ACh (30 nM) using enzyme immunoassay kits (Cayman Chemical, USA).

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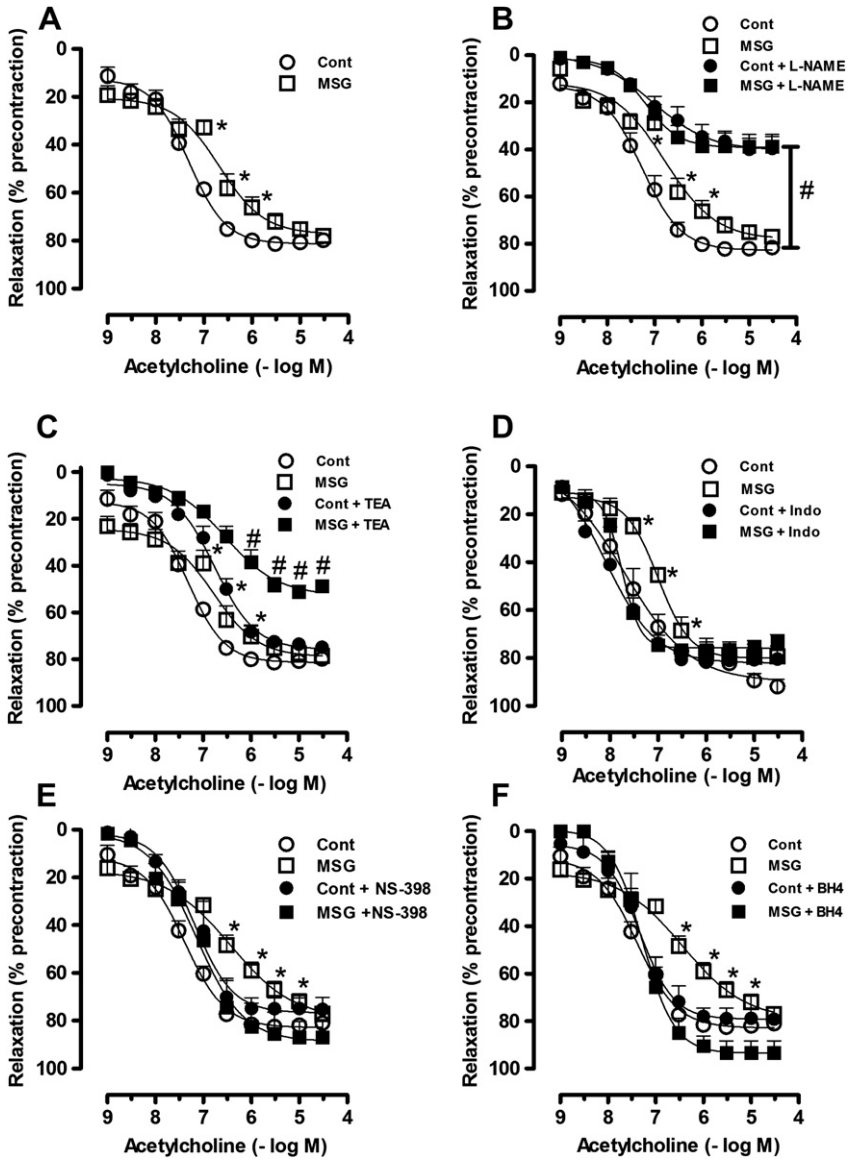

Figure 2 Concentration-response curves to acetylcholine in mesenteric arteriolar beds from control (Cont) and monosodium glutamate (MSG) rats in the absence (A) or presence of L-NAME (B, 100 μM), TEA (C, 2 mM), indomethacin (D, Indo 10 μM), NS-398 (E, 1 μM) or tetrahydrobiopterin (F, BH_4, 1 μM). Data are expressed as mean ± SEM of relaxation measured by percentage of contraction reduction. $n = 7–9$/group. *$P < 0.05$ vs. control. #$P < 0.05$ vs. MSG.

Measurement of NO production and ROS generation in mesenteric arterioles

NO production was determined using 4,5-diaminofluorescein diacetate (DAF-2) [19]. Mesenteric arterioles were dissected and embedded in freezing medium. Transverse arteriolar cryostat sections (20 μm) were collected on glass slides and incubated at 37 °C with 8 μM DAF-2 in phosphate buffer (0.1 M) containing $CaCl_2$ (0.45 mM). After 30 min, the sections were stimulated with ACh (100 μM) in the absence or presence of BH_4 (1 μM), as previously described [15]. Digital images were collected on an epifluorescence microscope (Carl Zeiss, Germany) and analyzed by measuring the mean optical density of the fluorescence of the endothelium.

ROS generation was determined by hydroethidine assay [20]. Transverse mesenteric arterioles were obtained as described for NO production and incubated at 37 °C with hydroethidine (2.5 μM) in phosphate buffer (0.1 M). Digital images were collected and the mean optical density of the fluorescence in the vessel wall was measured. To evaluate the effect of superoxide dismutase (SOD) and the participation of NOS in the ROS generation, mesenteric arterioles from MSG rats were treated with SOD (150 IU/mL) and L-NAME (10 μM) for 30 min before the tissues were frozen.

Western blotting

For analysis of eNOS and COX-2 protein expression, proteins (60 μg) extracted from mesenteric arterioles were separated by electrophoresis and Western blots were performed as previously described [21]. Antibodies were as follows:

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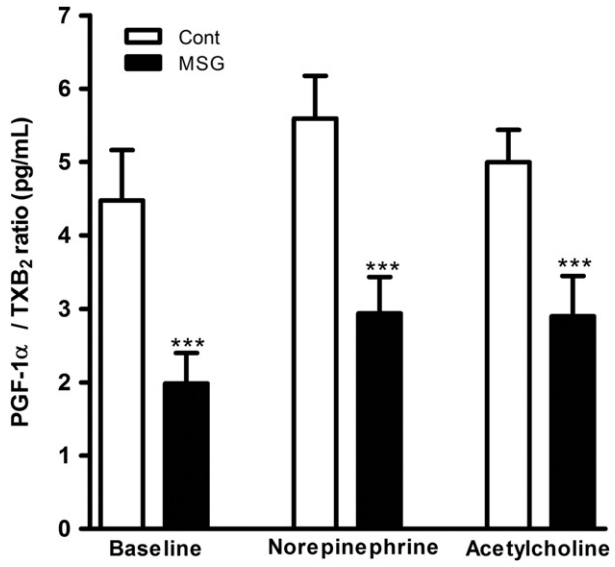

Figure 3 Basal, norepinephrine- and acetylcholine-induced release of 6-keto-PGF-1α and TXB2 from the mesenteric arteriolar bed perfusate of control and MSG rats. Data are expressed as mean $\pm$ SEM of 6-keto-PGF-1α and TXB2 levels ratio. $n = 8-12$/group. ***$P < 0.001$ vs. control.

anti-eNOS (1:1000; BD Transduction Laboratories, USA), COX-2 (3 μg/mL, Upstate, USA) or α-actin (1:5000; Sigma–Aldrich, Germany). The secondary antibodies used were as follows: anti-mouse IgG (1:1500; Bio-Rad, USA) for eNOS and α-actin and anti-goat IgG (1:5000; Jackson Immuno Research, USA) for COX-2.

Drugs

The following drugs were used: MSG, NE, ACh, BH_4, desipramine, SNP, indomethacin, L-NAME, SOD, and TEA (Sigma Chemical, USA); hydroethidine (Polysciences, USA) and DAF-2 (Alexis, USA).

Data analyses

Contraction is expressed as the KCl- and NE-induced perfusion pressure subtracted from the baseline pressure, and vasodilatation is represented as a percentage of the maximal response to NE. EC_{50} and the maximum response (R_{MAx}) were calculated by non-linear regression analysis. Data are represented as mean $\pm$ SEM and were compared by Student's *t*-test or one-way ANOVA when appropriate. $P < 0.05$ was considered significant.

Results

General characteristics of MSG rats

MSG rats displayed higher Lee index, fat mass, serum triglycerides and low density lipoprotein (LDL) cholesterol. In addition, enhanced HOMA-IR index, decreased kITT and hyperinsulinemia were found in MSG rats. No difference in BP levels was found between the groups (Table 1).

Vascular reactivity in the mesenteric arteriolar bed

Similar basal perfusion pressure was found in preparations from control and MSG rats. Treatment with L-NAME, BH_4, TEA, indomethacin or NS-398 did not alter the basal perfusion pressure in either groups. There was no difference in the KCl-induced contraction between the two groups (mmHg, control = 79.8 $\pm$ 2.9, MSG = 83.2 $\pm$ 2.1), however, the response to NE was significantly increased in MSG rats (Fig. 1A). Perfusion with L-NAME enhanced the responses to NE in control rats, which developed contraction levels similar to those exhibited by MSG rats. L-NAME further increased the NE-induced contraction in MSG rats (Fig. 1B). While TEA had no effect on the NE response in control rats, it markedly increased the vasoconstriction in MSG rats (Fig. 1C). There was no difference in NE responses between control and MSG rats after perfusion with indomethacin or NS-398 (Fig. 1D).

MSG rats displayed reduced sensitivity (lower pD_2) to ACh (Fig. 2A). The maximal response to ACh was significantly reduced in both groups after perfusion with L-NAME (Fig. 2B). Although the ACh response in control rats was not altered by TEA, the relaxation in MSG rats was significantly reduced after perfusion with this agent (Fig. 2C). Perfusion of the MAB of MSG rats with BH_4, indomethacin or NS-398 corrected the reduced sensitivity to ACh (Fig. 2D).

The sensitivity to SNP was higher in preparations from MSG rats (pD_2, control = 7.0 $\pm$ 0.1, MSG = 7.7 $\pm$ 0.2, $P < 0.01$).

Prostanoid release from the mesenteric arteriolar bed

The PGI_2/TXA_2 ratio obtained in unstimulated, NE- and ACh-stimulated preparations was significantly reduced in MSG rats when compared to control rats (Fig. 3).

Nitric oxide production and reactive oxygen species generation in mesenteric arterioles

Reduced basal and ACh-stimulated NO production were found in arterioles from MSG rats. The addition of exogenous BH_4 to preparations of MSG rats fully corrected the Ach-stimulated NO production (Fig. 4).

Arterioles from MSG rats displayed increased ROS generation (Fig. 4). Incubation with L-NAME or SOD reduced the ROS generation to values similar to those of control rats.

Protein eNOS and COX-2 expression

The protein expression of eNOS and COX-2 was increased in mesenteric arterioles from MSG rats (Fig. 5).

Discussion

Although obesity is one of the most important risk factors for cardiovascular disease [1,2], the mechanisms by which this condition affects the development of vascular dysfunction are still poorly understood. Considering that endothelial dysfunction has also been linked to type 2

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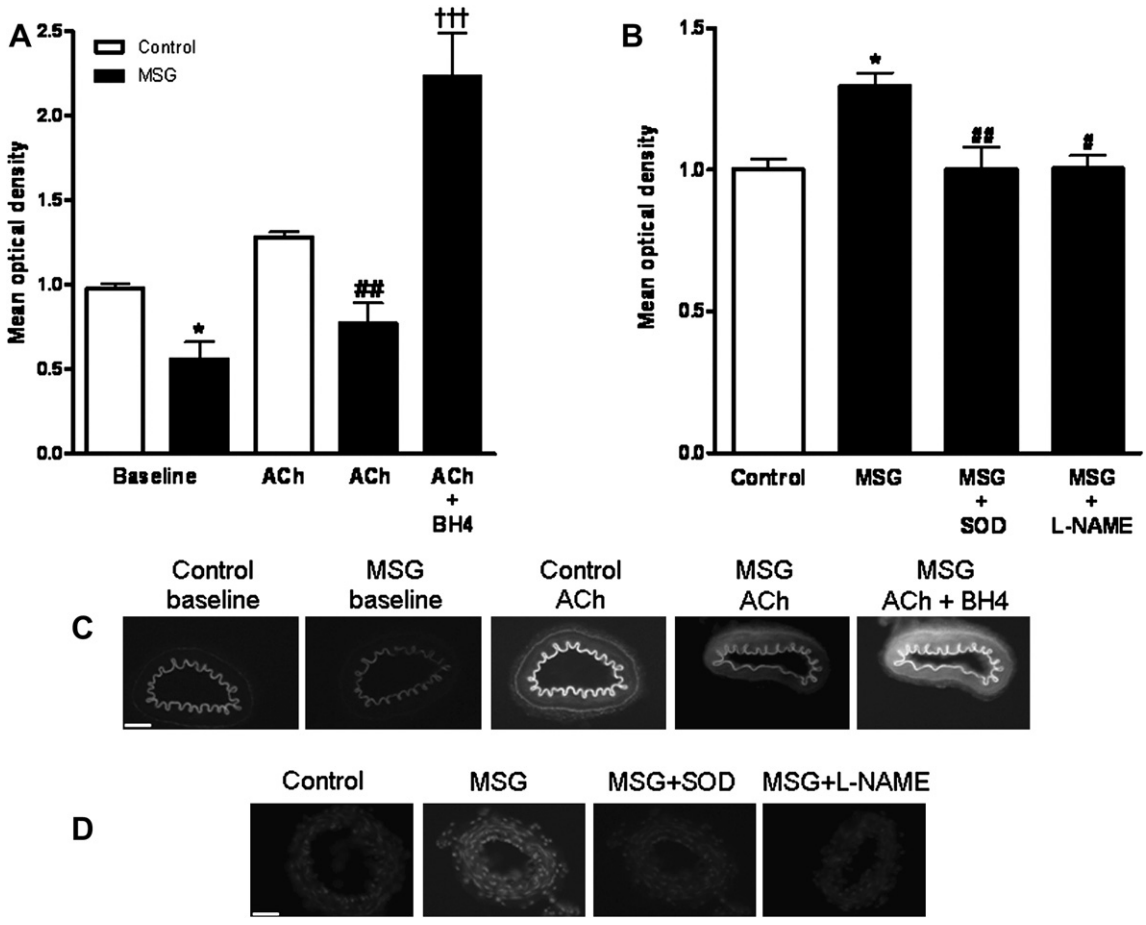

Figure 4 NO production and reactive oxygen species generation in mesenteric arterioles from control and MSG rats. (A)-Bar graphs show NO production measured by DAF-2 fluorescence. *$P < 0.05$ vs. control in basal condition. #$P < 0.05$ vs. control in ACh-stimulated condition. †††$P < 0.001$ vs. MSG stimulated by ACh. (B)-Reactive oxygen species generation, measured as hydroethidine-positive nuclei fluorescence. ***$P < 0.001$ vs. control; #$P < 0.05$ vs. MSG; #$P < 0.01$ vs. MSG. (C)-Representative fluorographs of transverse sections of arterioles with DAF-2 fluorescence. D- Representative fluorographs of transverse sections of arterioles with hydroethidine-positive nuclei. Data are expressed as mean ± SEM of the mean optical density of the fluorescence. $n = 5–6$/group. Scale bar: 20 μm.

diabetes [22] and hypertension [23], the current investigation attempted to minimize these confounding factors by using the MSG-induced obesity model. This model exhibits most features observed in human obesity (abdominal obesity, insulin resistance, hyperinsulinemia, and lipid profile abnormalities); however, type 2 diabetes and hypertension are absent in rats of the age used in this study.

Our control group consisted of rats injected with 0.9% saline solution, the MSG vehicle, instead of hypertonic solution, that is considered to be a better control for MSG injection [13,24]. Our choice is based on the fact that MSG rats display all of the features associated with human obesity when compared with control rats receiving either 0.9% saline ([25,26], present data) or hypertonic solution [13,24].

Although the present study does not allow us to establish the main factor contributing to vascular dysfunction in MSG rats, our results indicate that fat accumulation and insulin resistance contribute to alterations in microvascular reactivity in obesity independently of the presence of type 2 diabetes or hypertension. In fact, there are evidences showing that visceral adipocytokines regulate insulin sensitivity and vascular function [27]. Previous studies have shown that MSG rats display increased adipocytokine levels [28], which could provide a link between insulin resistance and the development of microvascular dysfunction in this model. Moreover, the development of hyperleptinemia, hyperinsulinemia, insulin resistance, and adiposity in MSG rats has been shown to be dependent on glucocorticoids [29]. Therefore, enhanced hypothalamo-pituitary–adrenal axis activity and increased levels of circulating corticosterone promoted by effects of MSG on ARC neurons might also link metabolic and vascular alterations in this model.

Modulation of angiogenesis may have the potential to impair adipose tissue development and, consequently, the development of obesity [30,31]. As the endothelium is important for the proper angiogenic response [30], we speculate that endothelial dysfunction contributes to the development of obesity in MSG rats by causing an increase in angiogenesis. Because insulin signaling plays an important role in maintaining endothelial function [15], pre-existing alterations in insulin signaling can contribute to alterations in angiogenic factors. Therefore, increased angiogenesis as a consequence of vascular dysfunction and

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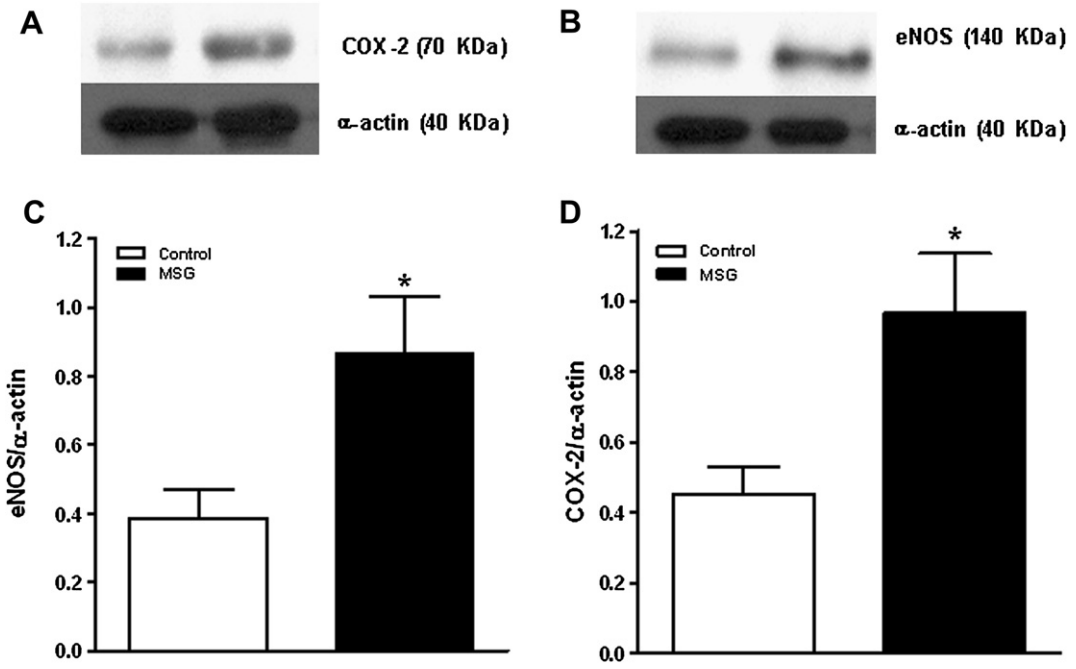

Figure 5 Representative Western blot for eNOS (A) and COX-2 (B) in mesenteric arterioles from control and MSG rats. Panels (C) and (D) show densitometric analysis of the Western blot for eNOS and COX-2 protein expression, respectively. Data are expressed as mean ± SEM of the ratio between the signal for eNOS or COX-2 protein and the α-actin signal. *$P < 0.05$ vs. control. $N = 5$/group.

insulin resistance might be factors contributing to the development of obesity in MSG rats.

We demonstrated increased vasoconstriction in response to NE along with decreased vasorelaxation in response to ACh in MSG rats. One mechanism that could account for these alterations is a change in receptor density. Because MSG rats display decreased catecholamine production [32] and hyperactivity of the parasympathetic nervous system [33], we suggest that modulation of the density of adrenergic and cholinergic receptors plays a role in the vascular dysfunction of MSG rats.

The increased NE-induced response in MSG rats cannot be related to alterations in the contractile apparatus of the vascular smooth muscle, because no difference was found in the response to KCl, a receptor-independent contractile agent. Furthermore, endothelium-dependent vasodilatation, which was tested with ACh, was impaired in MSG rats, indicating endothelial dysfunction. Similar results have been found in models of obesity with hyperglycemia or hypertension [34,35]. Therefore, we suggest that endothelial dysfunction precedes the development of hypertension and type 2 diabetes in obesity.

It is well known that the endothelium modulates the vascular tonus by releasing EDRFs and EDCFs [36]. Since NO is known to modulate NE- and ACh-induced responses [23], we determined the generation of and effects of NO in MSG rats. NO production, measured by DAF-2 fluorescence, is reduced and may contribute to reduced vasodilatation in MSG rats.

ROS generation can reduce endothelium-dependent vasodilatation by impairing NO bioavailability. One source of ROS is uncoupled eNOS. eNOS uncoupling is a process in which eNOS generates superoxide (O_2^-) when the concentration of either L-arginine, the substrate of NOS, or BH_4, a cofactor of the enzyme, is depleted [37]. Recent studies point to a crucial role of eNOS as a O_2^--producing enzyme in hypertension [37] and type 2 diabetes [15]. Because MSG rats showed enhanced ROS generation along with reduced NO production, we determined whether MSG-induced obesity promotes uncoupling of eNOS. Incubation of arterioles from MSG rats with either SOD, an O_2^- scavenger, or L-NAME, a NOS inhibitor, reduced ROS generation, suggesting a specific role for eNOS in O_2^- production. In addition, the BH_4-mediated correction of both the impaired ACh-induced relaxation and the reduced NO production further confirms that eNOS uncoupling as a consequence of BH_4 deficiency contributes to vascular dysfunction in MSG rats.

Because NO-dependent relaxation and NO production were decreased in MSG rats, we measured eNOS protein levels. Although decreased endothelium-dependent relaxation and NO production were found, eNOS protein expression was increased. These results are in agreement with another report showing that in type 2 diabetes, the expression of eNOS is paradoxically increased rather than decreased [38]. This indicates that the decreased NO-dependent relaxation in MSG rats is not due to decreased expression of eNOS but instead is related to the decreased ability of the enzyme to produce NO.

Although NO is considered the main mediator of endothelium-dependent relaxation, PGI_2 and EDHF are also important regulators of vascular reactivity, particularly in resistance vessels. Although the mechanism of vasodilatation induced by the different EDRFs differs, each individual mediator possesses the capacity to interact with components involved in the synthesis/activation of other mediators and control their activity. There is clear evidence that EDRFs work cooperatively in a complex but integrated manner to maintain homeostasis of the vasculature [36]. The fact that

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K^+ channel inhibition by TEA potentiated the response to NE and reduced the response to ACh only in MSG rats indicates that membrane hyperpolarization plays a major role in vasoconstriction and vasodilatation in the MAB.

The balanced release of PGI_2 and TXA_2 has an important role in vascular homeostasis [39]. TXA_2/PGH_2 receptor expression is enhanced in obesity-associated hypertension [40]. To our knowledge, however, there are no reports showing the vascular production of these prostanoids in obesity. We found a reduced PGI_2/TXA_2 ratio in basal, NE- and ACh-stimulated conditions in the MAB of MSG rats. The enhanced vasoconstriction as well as the reduced vasodilatation in MSG rats seems to be associated with an increased conversion of arachidonic acid to prostaglandins by COX, because the presence of indomethacin reversed these alterations.

Two isoforms of COX have been described. COX-1 is expressed constitutively, while COX-2 expression is induced under inflammatory conditions. In humans, an increase in COX-2 activity is associated with inflammatory conditions related to vascular diseases [39]. The finding of enhanced protein expression of COX-2 and the correction of vascular reactivity to NE and ACh after inhibition of this enzyme explain the altered profile of prostanoid release in the MAB from MSG rats; this finding may provide an additional mechanism for vascular dysfunction in obesity.

There are controversies in the literature about the influence of obesity on endothelium-independent relaxation. Most studies demonstrate impaired endothelium-dependent vasodilatation without alteration of endothelium-independent vasodilatation in obesity associated with co-morbidities [41]. However, a reduced vasodilator response to SNP, an endothelium-independent agent, was found in the arterioles of skeletal muscle from hypertensive diabetic obese Zucker rats [42]. In contrast, we found an increased sensitivity to SNP in MSG rats. We hypothesize that, in conditions where NO production or bioavailability is impaired, a compensatory hypersensitivity of smooth muscle to NO occurs. Data from Moncada's group [43] support this hypothesis. Using aortic rings treated with NOS inhibitors, an increased SNP response was shown, indicating that in the absence of endogenous NO release, a hypersensitivity of the vascular smooth muscle to exogenous NO occurs.

In summary, we showed that obesity impairs the microvascular reactivity independent of other risk factors, such as type 2 diabetes and hypertension. Reduced NO bioavailability and increased eNOS-generated ROS play a critical role in the development of vascular dysfunction. The imbalanced profile of prostanoid release via COX-2 also contributes to these alterations. Increased membrane hyperpolarization and hypersensitivity of the smooth muscle to NO may be mechanisms to compensate for the reduced NO production.

Our results contribute to the understanding of the mechanisms involved in vascular alterations in obesity, which may contribute to the development of hypertension and type 2 diabetes in obese individuals.

Conflict of interest

None declared.

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