



Adrenal medullary function and expression of catecholamine-synthesizing enzymes in mice with hypothalamic obesity

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Abstract

The mechanisms underlying the onset of obesity are complex and not completely understood. An imbalance of autonomic nervous system has been proposed to be a major cause of great fat deposits accumulation in hypothalamic obesity models. In this work we therefore investigated the adrenal chromaffin cells in monosodium glutamate (MSG)-treated obese female mice. Newborn mice were injected daily with MSG (4 mg/g body weight) or saline (controls) during the first five days of life and studied at 90 days of age. The adrenal catecholamine content was 56.0% lower in the obese group when compared to lean controls ($P < 0.0001$). Using isolated adrenal medulla we observed no difference in basal catecholamine secretion percentile between obese and lean animals. However, the percentile of catecholamine secretion stimulated by high K^+ concentration was lower in the obese group. There was a decrease in the tyrosine hydroxylase enzyme expression (57.3%, $P < 0.004$) in adrenal glands of obese mice. Interestingly, the expression of dopamine β -hydroxylase was also reduced (47.0%, $P < 0.005$). Phenylethanolamine N-methyltransferase expression was not affected. Our results show that in the MSG model, obesity status is associated with a defective adrenal chromaffin cell function. We conclude that in MSG obesity the low total catecholamine

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content is directly related to a decrease of key catecholamine-synthesizing enzymes, which by its turn may lead to a defective catecholamine secretion.

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Introduction

Obesity is a disease characterized by an excess of body fat that has been growing fast worldwide, from children to adulthoods (Inoue and Bray, 1979; Popkin and Doak, 1998; Ebbeling et al., 2002; Rossner, 2002). An imbalance between intake and expenditure of energy is a central factor in the etiology of obesity (Inoue and Bray, 1979; Flatt, 1995) as well the quality of the diet (Storlien et al., 1991). The hypothalamus plays a pivotal role in the control of food intake and energy homeostasis (Woods et al., 1985; Lawrence et al., 1999; Schwartz et al., 2000). The destruction of specific hypothalamic centers leads to the classically defined hypothalamic obesity (Tucker et al., 1965; Bray et al., 1981). Neonatal administration of monosodium glutamate (MSG) produces lesions in arcuate nucleus and median eminence of hypothalamus (McBride et al., 1976; Leigh et al., 1992). After repeated high doses of MSG, mice become obese in adulthood, as attested by increased Lee index and adipocyte hypertrophy (Fabres-Machado and Saito, 1995; Dolnikoff et al., 2001). These animals also present several neuroendocrine dysfunctions as decreased growth hormone secretion and hyperinsulinaemia (Nemeroff et al., 1978; Scallet and Olney, 1986; Maiter et al., 1991; Fabres-Machado and Saito, 1995; Kaufhold et al., 2002), without hyperphagia (Bunyan et al., 1976). On the other hand animals with electrolytic lesion in the ventromedial hypothalamus (VMH) are obese being hyperphagic (Baile et al., 1970; Bray et al., 1981) but obesity can occur even without increase in food intake (Han and Frohman, 1970). Moreover, results with mutant mice indicate that hyperphagia is not able to rapidly induce obesity without dysfunction in the sympathetic nervous system (SNS) (Nonogaki et al., 1998; Nonogaki, 2000). Actually, it has been proposed that autonomic nervous system plays an important role in the obesity development (Bray et al., 1981). Nevertheless, despite obesity had been usually associated with reduced SNS activity (Bray, 1991; Leigh et al., 1992; Young and Macdonald, 1992) confronting results had been found (Rumantir et al., 1999).

Notwithstanding other factors should be involved, the adrenal catecholamines could play an important role in the obesity onset. They, namely epinephrine and norepinephrine, have relevant participation in the energetic metabolism (Astrup et al., 1990; Scheurink and Ritter, 1993; van Dijk et al., 1995) and defective catecholamine synthesis and secretion may contribute to the development and/or maintenance of obesity (Young, 2002). The biosynthesis of catecholamines is accomplished within adrenal chromaffin cells by a sequential series of reactions where four enzymes are involved (Kumer and Vrana, 1996): tyrosine hydroxylase (TH, EC 1.14.16.2), the first enzyme of catecholamine biosynthesis pathway; dopa-decarboxylase; dopamine β -hydroxylase (DBH, EC 1.14.17.1); and phenylethanolamine N-methyltransferase (PNMT, EC 2.1.1.28), enzyme that converts norepinephrine into epinephrine.

The aim of our work was to study the adrenal chromaffin cell function in hypothalamic obesity. We firstly evaluated the catecholamine content in adrenal glands and catecholamine secretion from isolated

adrenal medulla of MSG-obese female mice. As we found impairment in both parameters we analyzed the expression of enzymes involved in catecholamine biosynthesis TH, DBH and PNMT. The obtained data provide evidence that the low catecholamine content is due to a decrease in TH and possibly DBH protein expression.

Materials and methods

Animals

Gestating Swiss mice were obtained from Central Vivarium of State University of Maringá and placed in an environmentally controlled room (23 ± 3 °C and 12 hour light/dark photocycle). All animals received water and complete commercial chow (Nuvital™, Colombo, PR, BR) at ease. During the first five days of life the newborn mice (six suckling by nurse) received a subcutaneous simple injection of MSG in the cervical region (4 mg/g body weight daily). Control group received equal volume of saline isosmotic to the MSG solution. At 21 days old, pups were weaned. Only female mice were used for the protocols. At 90 days old, mice were weighted and killed by cervical dislocation. The Lee index was calculated by the ratio (body weight^{1/3} (g)/nasoanal length (cm)) \times 1000 (Bernardis and Patterson, 1968). Adrenal glands were removed and cleaned of fat tissue under a stereomicroscope with ophthalmologic scissors. During handling, glands were maintained in standard Krebs-Hepes solution on ice bath. All protocols were approved by the ethic committee of the State University of Maringá.

Catecholamines assays

The total catecholamines—epinephrine and norepinephrine—were quantified by employing the trihydroxyindole fluorescence method (Kelner et al., 1985). The parameters used in the fluorometer comprised 420 nm to excitation and 510 nm to emission. The assay was done with 75 μ l of the incubation solutions and supernatant of homogenized samples. For total tissue catecholamine content, right adrenal glands were homogenized in 220 μ l of 10% acetic acid using an ultrasonic processor and centrifuged at 10,000 g for 1 min. Results were obtained by plotting the values into a linear regression of standard epinephrine curve. Protein concentration was determined by the Bradford assay. For catecholamine secretion protocols, left adrenal glands were dissected and had their medullae removed in toto with microscissors. After dissection the medullae were impaled with 0.3 mm-diameter stainless needle for better manipulation and maintained for 1 hour at room temperature in standard Krebs-Hepes solution composed of (mM): Cl⁻, 154.3; Na⁺, 143.4; Ca²⁺, 2.5; Mg²⁺, 1.18; (SO₄)²⁻, 1.2; K⁺, 5.9; glucose, 11.1; Hepes, 25; and bovine serum albumin 0.5%; pH 7.4. Protocols were carried out in 96 wells tissue culture plate that contained 200 μ l of Krebs solution. Medullae were stimulated for 10 minutes in Krebs-Hepes solutions containing 50 mM of K⁺ (stimulated secretion) or maintained in a standard Krebs-Hepes solution (basal secretion). In the experiments with excess of K⁺ equivalent concentration of Na⁺ was removed to maintain the osmolarity. At the end of the experiments the medullae were packed in eppendorf tubes containing 220 μ l of 10% acetic acid, homogenized and centrifuged. The solutions in the wells were acidified with 20 μ l of concentrated acetic acid.

Table 1
Biometric parameters of control and MSG-obese female mice

	Lee index	Perigonadal fatty (g)	Retroperitoneal fatty (g)
Control	329.8 ± 1.4 (30)	0.9 ± 0.05 (8)	0.4 ± 0.04 (9)
MSG mice	371.9 ± 3.8 (30)***	1.8 ± 0.2 (8)**	0.8 ± 0.06 (9)***

Results are mean ± SEM (n).

** $P < 0.01$.

*** $P < 0.0001$.

Western blot analyses

Both glands were used for TH, DBH and PNMT immunoblotting. After removed and cleaned, glands were placed immediately in liquid nitrogen. Further, glands were homogenized with 100 µl of tris-HCl buffer, containing protease inhibitors (Cocktail tablets, Complete™, Boehringer Mannheim GmbH, Mannheim, GE). Protein concentration was determined by the Bradford assay. Supernatants of homogenized samples containing 20 µg for TH and 40 µg of proteins for DBH and PNMT were diluted in equal volume of Laemmli buffer and heated up to 95 °C for 5 minutes. Proteins were separated on 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane (ProTrans, Keene, NH, USA). Non-specific binding sites of the membranes were blocked by incubation in 5 ml of TBS with 5% non-fat dry milk and 0.15% Tween-20 over night (TH and DBH, at 4 °C) or for 1 h (PNMT, at room temperature). Afterwards, membranes were washed with TBS and incubated with primary antibodies: monoclonal mice anti-TH diluted 1:1000 for 1 h at room temperature; and polyclonal rabbit anti-DBH (Eugene Tech International, Ridgefield Park, NJ, USA) diluted 1:1000; and polyclonal rat anti-PNMT (Chemicon, Temecula, CA, USA) diluted 1:1250-both incubated over night, at 4 °C. Later on, membranes were washed with TBS and incubated with adequate secondary antibodies at concentrations of 1:10,000 for TH and 1:1000 for DBH and PNMT, for 1 hour at room temperature. At the end, protein bands were visualized by chemiluminescence using the Enhanced Chemiluminescence's system (ECL™, Amersham, Buckinghamshire, UK) and horseradish peroxidase label followed by short exposure (5 to 20 s) to autoradiographic film (Kodak Bio Max Film, Kodak, Rochester, NY, USA).

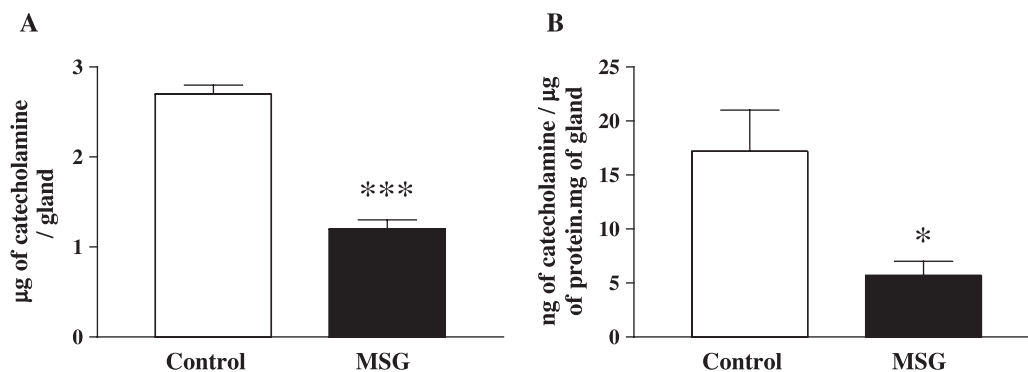


Fig. 1. Catecholamine content from control and MSG-obese mice. Mice treated neo-natally with MSG had decreased catecholamine content in the adrenal glands (A). The result is confirmed when values are plotted relative to the protein content (B). *** $P < 0.0001$, * $P < 0.05$.

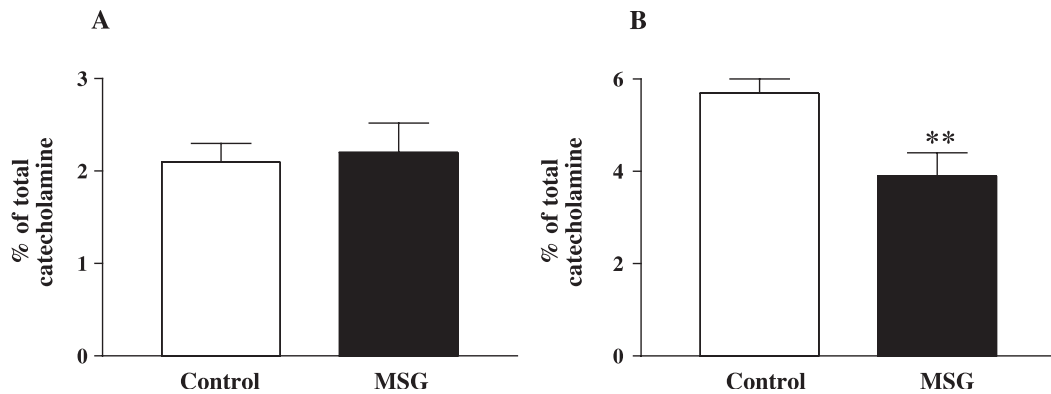


Fig. 2. Catecholamine secretion from control and MSG-obese female mice. There were no differences on the basal (non-stimulated) catecholamine secretion percentile (A). On the other hand, MSG-obese female mice presented decreased catecholamine secretion stimulated by high (50 mM) potassium concentration (B). ** $P < 0.01$.

The immunoreactive bands were evaluated by measuring the area and its density with the program ImagePro (Media Cybernetics, Maryland, USA).

When not specified reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis

All results are presented as mean \pm SEM. Student's t-test was used for group comparison. $P < 0.05$ was considered statistically significant.

Results

Biometric parameters of 90 days old animals are summarized in Table 1. MSG-treated female mice became obese as indicates the Lee index ($P < 0.0001$). Large fat deposits, a central characteristic of obesity status, were also recorded. Increased perigonadal ($P < 0.001$) and retroperitoneal fatty weights ($P < 0.0001$) in obese group confirmed the efficiency of MSG treatment to induce obesity.

Fig. 1 shows total catecholamine content in adrenal glands of obese and lean animals. MSG-obese female mice had a reduction of 56.0% ($P < 0.0001$) in the catecholamine content ($1.2 \pm 0.1 \mu\text{g/gland}$, $n = 8$) in comparison with control animals ($2.7 \pm 0.1 \mu\text{g/gland}$, $n = 8$) (Fig. 1A). This difference is

Table 2
Catecholamine-synthesizing enzyme expression from control and MSG-obese female mice

	Tyrosine hydroxylase	Dopamine β -hydroxylase	Phenylethanolamine N-methyltransferase
Control	48235.1 \pm 7847.8 (6)	90043.7 \pm 11485.8 (3)	60350.3 \pm 10406.9 (3)
MSG mice	20590.9 \pm 1565.1 (7)**	47689.1 \pm 3527.7 (5)**	61450.3 \pm 6493.2 (3)

Results are mean \pm SEM (n). Results are in arbitrary units.

** $P < 0.01$.

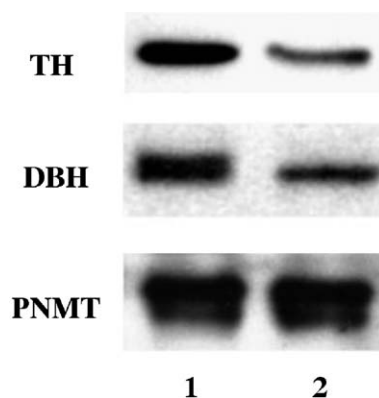


Fig. 3. Catecholamine-synthesizing enzyme expression from control (lane 1) and MSG-treated obese group (lane 2). Cellular protein (10 μ g for TH, and 20 μ g for DBH and for PNMT) was subject to Western blotting analyses of each enzyme. Shown is a representative blot of at least three sets of independent experiments each one in duplicate.

confirmed when the results are expressed as a ratio of catecholamine content per total protein content in the gland (5.7 ± 1.3 and 17.2 ± 3.8 ng of catecholamine/ μ g of protein/mg of gland, $n = 8$, $P < 0.02$) (Fig. 1B), although no significant differences were found in total protein content (not shown).

There was no significant difference in the basal catecholamine secretion percentile between MSG-obese and lean mice, as shown in Fig. 2A. On the other hand, the response to stimulation with 50 mM of potassium was impaired in the obese group, as shown in Fig. 2B. Adrenal medulla from obese animals secreted $3.9 \pm 0.5\%$ of their total catecholamine content while lean animals secreted $5.7 \pm 0.3\%$ ($n=8$, $P < 0.009$).

Expression of catecholamine-synthesizing enzymes in adrenal glands from obese and lean animals is presented in Table 2 and representative blots in Fig. 3. A reduction of 57.3% ($P < 0.004$) in the TH expression was found in the obese group comparing with the control group. Similarly, the expression of DBH was also affected. The obese group presented a reduction of 47.0% ($P < 0.005$) of the enzyme expression in comparison with the control group. The PNMT expression was not affected. It is interesting to note the close correlation between the extension of catecholamine decrease (reduced 56.0% in the obese group) and the extension of TH enzyme expression decrease (reduced 57.3% in the obese group).

Discussion

We have shown in this work that stimulated catecholamine secretion is impaired in adrenal medulla and that adrenal catecholamine stores are decreased in MSG-obese female mice. Our results strongly suggest that this reduction in catecholamine stores is caused primarily by decreased expression levels of TH and DBH. We provided, what is to our knowledge, the first detailed report of TH and PNMT enzyme expression in adrenal gland of obesity animal model. We also show for the first time that catecholamine secretion is impaired in MSG-obese female mice, results corroborated by our previous studies in MSG-obese male mice (Martins et al., 2001). The advantage of the methodology used by us, i.e. isolated adrenal medulla and gland, is that this approach avoids possible external interferences as that can be found

in *in vivo* measurements of catecholamines, such as release of catecholamines from sympathetic neurons, catecholamines clearance and turnover, tissue distribution, and others. Thus the results presented here can be pinpointed in a strict cellular and molecular level.

Adrenal medullary secretion of catecholamines, under integrated *in vivo* control, is far a complex mechanism. It involves several signal molecules and second messengers (Burgoyne, 1991; Morita et al., 1997; Osipenko et al., 2000) and the secretion behavior depends on the species (Malhotra et al., 1998; Abad et al., 1992; Finnegan et al., 1996), health status (Del Rio, 2000; Cryer et al., 2003) and the cross talking among various signaling pathways (Morita et al., 1997; Osipenko et al., 2000). However, the basic mechanism for exocytosis in the stimulated secretion seems very preserved in the different species. Following membrane depolarization, the intracellular calcium levels raise and the membrane fusion between vesicle and plasma membrane is accomplished by an intricate mechanism where several proteins are involved (Akaike et al., 1990; Graham and Burgoyne, 2000; Quetglas et al., 2002). This membrane depolarization can be mimetically done *in vitro* by the use of high potassium concentration in the medium (Akaike et al., 1990).

Our results are in accordance with previous findings on obese humans who have reduced plasma epinephrine levels under stimulation (Young and Macdonald, 1992), in spite of divergent reports showing no difference (Menozzi et al., 2002). They also provide new evidence that the basal catecholamine secretion pathway is not impaired in obese animals since the percentile of secretion is not different from lean controls. However, secretion could be lower due to the decrease in total catecholamine content (as discussed below). Defective basal catecholamine secretion has been cited as a controversial issue and both evidences for impairment and for no alteration in basal catecholamine secretion in obesity have been found (Young and Macdonald, 1992; Macdonald, 1995).

The heterogeneity of findings in catecholamine secretion and synthesis of the adrenomedullary system of obese humans and animals may be consequence of the gender, age, animal model and obesity subtype, methods of collect and measurement of the catecholamines, methods to stimulate the catecholamine secretion and data analysis (Young and Macdonald, 1992; Ravussin and Tataranni, 1996; Del Rio, 2000). Therefore, attention is necessary about extrapolations. Gender specific control to which the SNS is submitted is of special interest (Del Rio, 2000). For instance, MSG-obese female rats show a reduction of mRNA for TH and TH protein expression in hypothalamus while it is kept unchanged in males (Arbogast and Voogt, 1990). On the other hand, in rats fed a cafeteria diet only males and testosterone-treated females have an increase in hypothalamic TH mRNA (Plut et al., 2002). However, this gender mechanism does not appear to be operating in the adrenal medullary system of our MSG-obesity model (Martins et al., 2001).

Our results also show that the catecholamine secretion in obesity is impaired due in part to the reduced catecholamine content. It appears to be true when analyzing the secreted catecholamines percentile. The basal catecholamine secretion was the same in both lean and obese animals in percentile values, but it would be also right if we stated a reduced catecholamine secretion as the total catecholamine content is decreased in obese animals, i.e., the percentile values are the same but the overall amount of secreted catecholamines in this case is lower in obese mice. In other words, the results suggest that the secretory pathway is apparently normal but the quantity of catecholamine released is lower. In the case of stimulated catecholamine secretion not only the overall amount is diminished but also the percentile of secretion. It indicates an impairment of the mechanisms that lead to the secretion not present in the basal catecholamine secretion. We are thus tempted to speculate that the catecholamine secretion pathway (stimulated and non-stimulated secretion) might be differently affected

in hypothalamic obesity and to hypothesize that the basal secretion depends only on the quantal release which is decreased in obesity.

The adrenal catecholamine content in obesity animal model had been accessed before by Levin and collaborators (Levin et al., 1981). Four months-old male Zucker rats showed increased epinephrine content in adrenal glands and normal levels of norepinephrine. On the other hand, eight months-old rats presented decreased norepinephrine levels and normal levels of epinephrine. These results suggest a differential time-dependent alteration in the content of adrenal catecholamines. But the decrease in total catecholamine content predicted for the older Zucker rats is too small when compared with our results in MSG-obese female mice, reinforcing the importance of gender and/or obesity model. In the same work the enzymatic activities of TH, DBH and PNMT were assessed and also showed a time-dependent alteration. TH activity was increased in the younger and PNMT activity was increased in the older rats. DBH activity was decreased in both younger and older rats. The latter observation suggests that there is more than one control point for catecholamine biosynthesis, making DBH a suitable target to the factors that modulate it. However, the authors stated that the decrease in DBH activity was not a consequence of diminished levels of enzyme protein. Our results show a decrease in the TH enzyme expression as well as a decrease in the DBH enzyme expression in MSG-obese female mice. Therefore, although it would not be a surprise that DBH could play an important role in the regulation of catecholamine biosynthesis, in the MSG-obesity model the long term regulation of DBH appears to be a major feature. However, it is well accepted that TH is the rate limiting enzyme in the catecholamine biosynthetic pathway (Kumer and Vrana, 1996). Nevertheless in this view it should also be accepted that TH act as the limiting step in the catecholamine secretion and that the reduction in DBH, independently of its causes, has not a significant influence on the decrease in total catecholamine content, in pathological conditions it would not be completely right.

Several works focus on the relationship between obesity and SNS and different findings have been obtained (Young and Macdonald, 1992; Macdonald, 1995; Corry and Tuck, 1999). There are works showing a reduction in the SNS activity (Vander Tuig et al., 1982; Young and Macdonald, 1992), increase in the SNS activity (Troisi et al., 1991; Young and Macdonald, 1992; Yamakawa et al., 1995; Grassi et al., 1998; Carlson et al., 2000) and others showing no relation between obesity and defective SNS function (Young and Macdonald, 1992). Moreover, there is a well established relationship between the SNS activity and food intake (Ravussin and Tataranni, 1996). An important characteristic of the adrenal chromaffin cells is that they have the same embryological origin of sympathetic neurons (Anderson and Axel, 1986; Souto and Mariani, 1996) making them a useful model to study the SNS. However, differential pattern has been recorded involving the adrenal medullary cells and the SNS in obesity (Young and Macdonald, 1992). Additionally, there are reports showing SNS units regulated by independent ways (Jänig and McLachlan, 1992). Therefore it is not certain whether the sympathoadrenal system should be considered a unique, two or multiple functionally distinct entities, although the evidences indicate that it might be more than one (Jänig and McLachlan, 1992; Macdonald, 1995; Del Rio, 2000; Young, 2002).

Recently, conflicting to our results in MSG-obese mice, it was shown that epinephrine levels in plasma were found elevated in VMH-obese female rats (Valensi et al., 2003). This result indicates that adrenal catecholamines secretion is increased in VMH rats. Preliminary results obtained in our laboratory indicate that this opposite effect is probably due to the species studied rather than to the obesity model. The authors also speculated that this effect is possibly due to hyperinsulinaemia. However, hyperinsulinaemia is also a characteristic of MSG-obese mice (Fabres-Machado and Saito, 1995) and therefore, according to

our results, it is unlikely that insulin increases catecholamine secretion from adrenal medulla in MSG-obese mice. Furthermore, we have previously shown that insulin has no acute effect on catecholamine secretion from isolated adrenal medulla of rats (de Araujo e Souza et al., 1999). Insulin receptors have been found on adrenal medullary cell membrane and certainly have a physiological function (Dahmer and Perlman, 1988; Yamamoto et al., 1996). It has also been found that incubation of bovine chromaffin cells with insulin increases the catecholamine content, particularly that of norepinephrine rather epinephrine (Wilson and Kirshner, 1983). However, whether long term insulin exposures per se have an effect on the secretion of adrenal chromaffin cell from obese rats is still unknown.

In conclusion, our results show that there is a defective catecholamine biosynthesis in adrenal glands from monosodium glutamate-treated obese female mice and that it is due mainly to a decrease in the catecholamine-synthesizing enzymes expression tyrosine hydroxylase and, possibly, dopamine β -hydroxylase. Hence adrenal medulla from MSG-obese female mice secretes lower amounts of catecholamines, although the mechanisms of secretion impairment might have additional causes for the stimulated secretion. Moreover, taking into account the importance of adrenal medullary catecholamines in the catabolic process as lipolysis induction, increase of energetic expenditure and glycogen breakdown, we also conclude that the impairment of the catecholamine biosynthesis pathway can be an important factor in the hypothalamic obesity development and/or maintenance in rodents. Our results support the observations that there is a defective adrenal medullary activity associated with obesity and reassert the hypothesis that in hypothalamic obesity there is a reduction of the sympathoadrenal system activity.

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