HPA axis and vagus nervous function are involved in impaired insulin secretion of MSG-obese rats

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Abstract

Neuroendocrine dysfunctions such as the hyperactivity of the vagus nerve and hypothalamus–pituitary–adrenal (HPA) axis greatly contribute to obesity and hyperinsulinemia; however, little is known about these dysfunctions in the pancreatic β -cells of obese individuals. We used a hypothalamic-obesity model obtained by neonatal treatment with monosodium L-glutamate (MSG) to induce obesity. To assess the role of the HPA axis and vagal tone in the genesis of hypercorticism and hyperinsulinemia in an adult MSG-obese rat model, bilateral adrenalectomy (ADX) and subdiaphragmatic vagotomy (VAG) alone or combined surgeries (ADX–VAG) were performed. To study glucose-induced insulin secretion (GIIS) and the cholinergic insulinotropic process, pancreatic islets were incubated with different glucose concentrations with or without oxotremorine-M, a selective agonist of the M_3 muscarinic acetylcholine receptor (M_3 AChR) subtype. Protein expression of M_3 AChR in pancreatic islets, corticosteronemia, and vagus nerve activity was also evaluated. Surgeries reduced 80% of the body weight gain. Fasting glucose and insulin were reduced both by ADX and ADX–VAG, whereas VAG was only associated with hyperglycemia. The serum insulin post-glucose stimulation was lower in all animals that underwent an operation. Vagal activity was decreased by 50% in ADX rats. In the highest glucose concentration, both surgeries reduced GIIS by 50%, whereas ADX–VAG decreased by 70%. Additionally, M_3 AChR activity was recovered by the individual surgeries. M_3 AChR protein expression was reduced by ADX. Both the adrenal gland and vagus nerve contribute to the hyperinsulinemia in the MSG model, although adrenal is more crucial as it appears to modulate parasympathetic activity and M_3 AChR expression in obesity.

Key Words

- ▶ HPA axis
- ▶ PNS
- ▶ insulin secretion
- ▶ M_3 muscarinic receptor
- ▶ MSG rats

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Introduction

Metabolic derangements, especially during early life, are the major etiology of chronic metabolic diseases worldwide. Obesity that is associated with cardiovascular and type 2 diabetes causes a high incidence of comorbidities and death (Kelly *et al.* 2008). The origins of obesity and the role of the pancreatic β -cell as a hallmark of metabolism malfunction during the onset of obesity are not clear. Glucose homeostasis imbalance has a tight association with β -cell malfunction, producing hyperinsulinemia during fasting and feeding states (Shafir 1996).

Why is glucose-induced insulin secretion upregulated in obese human beings and experimental animal models? Several hypotheses have been proposed. One hypothesis is largely debatable: tissue insulin resistance provokes the oversecretion of insulin by β -cells (Grassioli *et al.* 2006). Hyperglycemia supervenes on increased insulin resistance and a lack of consequent insulin release increases, which cause a toxic effect on β -cell function, leading to a vicious cycle (Solomon *et al.* 2012). This effect, known as glucotoxicity, may itself disrupt glucose signal transduction in β -cells to stimulate insulin release (Bensellam *et al.* 2012). Moreover, other secretagogues are also crucial to increase plasma insulin. Pancreatic β -cells receive many neural terminals, including the autonomic nervous system (ANS) with their parasympathetic (PNS) and sympathetic (SNS) branches, which release acetylcholine (ACh) and noradrenaline (NA), respectively. Although ACh potentiates a glucose insulinotropic effect, NA blocks this effect (Campfield & Smith 1983). Cholinergic transduction in pancreatic β -cells mostly involves cholinergic muscarinic receptors (MACHR), which are constituted by four subtypes: M_1 , M_2 , M_3 , and M_4 . The cholinergic muscarinic insulinotropic effect lies mainly in the M_3 subtype of MACHR (M_3 AChR) (Boschero *et al.* 1995).

Studies have suggested that in obese human beings and animals, the ANS is imbalanced, showing high vagal activity (Weyer *et al.* 2001). In addition, hyperinsulinemia has been observed in obese individuals and is associated with the hyperactivity of the PNS by releasing ACh. Regarding this matter, subdiaphragmatic vagotomy (VAG) has been performed to attenuate the hyperinsulinemia in monosodium L-glutamate (MSG)-treated rats and other obese models such as Wistar fatty models (Yamatani *et al.* 1998, Balbo *et al.* 2000). The MSG rat is an interesting model that reconciles increased parasympathetic activity, hyperinsulinemia, and obesity, among other components of the metabolic syndrome, mimicking the

disorders observed in obese individuals (Hirata *et al.* 1997, Dolnikoff *et al.* 2001, Karlen-Amarante *et al.* 2012).

Pancreatic islets from MSG-obese rats incubated with carbachol, a cholinergic muscarinic agonist, showed lower insulin secretion than those from lean rats, demonstrating a weak cholinergic response (Balbo *et al.* 2002); however, M_3 AChR was shown to be highly expressed in the pancreatic islets from MSG-obese rats (Miranda *et al.* 2014).

However, in addition to PNS hyperactivity, dysfunction in the hypothalamus–pituitary–adrenal (HPA) axis is an important factor that contributes to metabolic syndrome (Wang 2005, Laborie *et al.* 2011). In fact, excessive blood levels of glucocorticoids have been associated with insulin resistance and visceral obesity (de Guia & Herzig 2015).

Lean adult rats treated with dexamethasone displayed hyperinsulinemia associated with high protein expression of M_3 AChR in the pancreatic islets (Angellini *et al.* 2010). Furthermore, ablation of the adrenal glands normalized the blood insulin levels of hypothalamic obese rats (Perello *et al.* 2003). Accordingly, the high activity of both the PNS and HPA axis is hypothesized to contribute to the disrupted regulation of insulin secretion in obesity. Thus, to test this hypothesis, we subjected the MSG-obese rat model to either vagotomy or adrenalectomy or to both surgeries to evaluate the contribution of the PNS and HPA axis for pancreatic β -cell dysfunction.

Materials and methods

Animals and obesity

All experiments were undertaken according to the norms established by the Brazilian Association for Animal Experimentation (COBEA) and were previously approved by the Ethical Conduct Committee on Animal Use in Experimentation (protocol number 147/2012).

Neonate male Wistar rats were subcutaneously injected during the first 5 days of life with MSG at a dose of 4 g/kg of body weight (BW) (Olney 1969, Hirata *et al.* 1997). Control animals received equimolar saline solution. All animals were weaned at 21 days of age. The animals were housed under controlled conditions in a 12 h light:12 h darkness cycle, with the lights switched on at 07:00–19:00 h and temperature at $21 \pm 2^\circ\text{C}$. Water and standard rodent chow (Nuvital, Curitiba, Brazil) were supplied *ad libitum*. Only males were used in the experimental protocols. MSG rats that reached at least a 2.5% increase in the Lee index compared with those treated with saline solution were chosen.

At 90 days of age, some of the rats from both groups, control and MSG, were used to evaluate body composition and metabolic parameters. The rats were anesthetized and killed to evaluate biometric and biochemical parameters to characterize the obesity induced by MSG. Other rats of the MSG group, randomly chosen, were submitted to bilateral subdiaphragmatic vagotomy (VAG), $n=20$, or to bilateral adrenalectomy (ADX), $n=30$, or both surgeries (ADX-VAG), $n=20$, whereas another batch of MSG rats were false operated (sham), $n=26$. All the rats that underwent the surgery procedures were kept in isolated cages during surgery rehabilitation for a period of 10 days. After the surgeries, 20% of death was recorded to the adrenalectomized and/or vagotomized rats and 30% to those submitted to both operations.

BW gain was evaluated by subtracting the weight of the animals 10 days after surgery to the weight before the surgery, and the food consumption was measured by weighing the remaining food, subtracting that amount from the ration offered to the animals the day before, and the values were expressed relative to 100 g of BW.

At 90 days of age, control and/or MSG rats that did not undergo an operation ($n=12$), and all rats at 100 days of age (operated or sham-operated under anesthesia, 45 mg/kg/BW thiopental) were killed by cervical dislocation. Then, the visceral fat pads were removed, washed, and weighed, and Lee index ($BW (g)^{1/3}/\text{nasal-anal length (cm)} \times 100$), which is employed as a predictor of obesity in MSG rodents (Bernardis & Patterson 1968), was calculated. Blood samples were collected to measure glucose and hormone concentrations, including insulin, leptin, and corticosterone concentrations.

Adrenalectomy

Under anesthesia using thiopental (45 mg/kg BW i.p.), MSG rats at 90 days of age underwent bilateral ADX. The surgery was performed using a dorsal approach. Sham-ADX was performed using the same approach, with only localization of the adrenals, leaving the glands intact. ADX rats were maintained on 0.9% NaCl to correct the aldosterone insufficiency throughout the 10 days following the operation (Su *et al.* 2015).

Vagotomy

Vagotomy and sham surgeries were performed in MSG animals, both intact and in ADX rats. Rats at 90 days of age after 12 h of fasting were anesthetized with thiopental (45 mg/kg BW i.p.). After laparotomy, both the dorsal and

subdiaphragmatic vagal trunks were identified from the esophagus and severed with the aid of a stereomicroscope (Balbo *et al.* 2007). In sham vagotomized rats, the vagus nerve was separated from the esophagus but was not cut. After surgery, on the 10th day and after a 12 h fasting, the animals were anesthetized with thiopental (45 mg/kg BW i.p.) and killed by decapitation. Using stomach food retention as a characteristic of vagotomy, 100-day-old animals from all the groups were observed. After killing the animals, the abdomens were opened and the stomachs were removed by severing the esophagus at one end and the pyloric sphincter at the other. The stomach was weighed, and the ratio of stomach weight to total BW was calculated. A confidence interval of 99% was employed to calculate the mean ratio for controls, and its upper limit was used as rejection criterion for vagotomy effectiveness (Bernstein & Goehler 1983, Balbo *et al.* 2002).

Intravenous glucose tolerance test

Under ketamine and xylazine anesthesia (3 and 0.6 mg/100 g of BW, respectively), a silicone cannula was implanted into the right jugular vein of MSG rats, either in operated or sham-operated rats at 100 days of age. The cannula was stabilized in the dorsal region of the neck. The cannula was previously treated with heparinized saline (50 IU heparin/mL of saline solution (NaCl, 0.9%)) to avoid blood clots. After a 12 h fast (19:00–07:00 h) and without anesthesia, a glucose load (1 g/kg BW) was infused through the cannula. Blood samples (350–400 μ L) were collected immediately before the glucose load (0 min) and at 5, 15, 30, and 45 min after the load (De Oliveira *et al.* 2013). Plasma obtained from the blood samples was stored at -20°C for subsequent determination of glucose and insulin concentrations.

Intraperitoneal insulin tolerance test

A batch of control and MSG rats had a cannula implanted into the right jugular vein as described above and were fasted for 6 h before an intraperitoneal insulin tolerance test (ipITT; 1 U/kg BW). As previously described, the same method of blood collection used in the intravenous glucose tolerance test (ivGTT) was used to perform the ipITT. The absorption rate of glucose by the tissue (rate constant for the disappearance of plasma glucose (K_{itt})) was calculated using the formula $0.693/(t_{1/2})$, which was already described (Lundbaek 1962). The plasma glucose $t_{1/2}$ was calculated from the slope of the least squares

analysis of the plasma glucose concentrations during the linear phase of the decline (De Souza *et al.* 2005).

Pancreatic islet isolation

Pancreatic islets were isolated from operated or sham-operated rats using a collagenase enzyme technique as previously described, with some adaptation (Gravena *et al.* 2002). At 100 days of age, after being anesthetized with 45 mg/kg BW thiopental, the rats were decapitated and the abdominal wall was cut open. Then, 8 mL of Hanks buffered saline solution (HBSS, (mmol/L): NaCl, 136.9; KCl, 5.4; MgSO₄·7H₂O, 0.81; Na₂HPO₄, 0.34; KH₂PO₄, 0.44; CaCl₂·2H₂O, 1.26; NaHCO₃, 4.16; glucose, 0.06; BSA, 15, and (O₂, 95% + CO₂, 5% mixed)/10 min, pH 7.4) containing collagenase type XI, 0.1% plus BSA, 5%, and HEPES, 0.6% (Sigma-Aldrich) was injected into the common bile duct. The pancreas was swollen with the collagenase solution and was quickly excised and incubated in a glass beaker for 17–18 min. The suspension was then discharged, and the precipitated islets were washed three times with HBSS. The islets were collected with the aid of a stereomicroscope. At least three rats from three different litters were used for each experimental procedure of each group of animals.

Insulin secretion stimulation

To adapt isolated pancreatic islets to a baseline glucose concentration (5.6 mmol/L), the islets were pre-incubated for 60 min in 1 mL of normal Krebs–Ringer solution ((mmol/L): NaCl, 115; NaHCO₃, 24; KCl, 1.6; MgCl₆·H₂O, 1; CaCl₂·2H₂O, 1; BSA, 15) and pH 7.4 containing 5.6 mmol/L glucose. To study glucose-induced insulin secretion, after the pre-incubation, a batch of pancreatic islets were incubated for a further 60 min in Krebs–Ringer solution containing different glucose ((mmol/L): 5.6, 8.3, and 16.7) concentrations. Another batch of pancreatic islets were incubated for a further 60 min in Krebs–Ringer solution containing different glucose (basal, 5.6 mmol/L) or (high 16.7 mmol/L) concentrations in the presence or absence of a selective M₃AChR agonist, oxotremorine-M (1 mmol/L), to evaluate the cholinergic muscarinic insulinotropic response.

Western blot analyses

Isolated pancreatic islet M₃AChR content from 100-day-old MSG rats, either operated or sham-operated, was

determined by immunoblotting. Three hundred islets of each experimental group were frozen at –80°C in HBSS to posterior sonication (two times, 10 s pulses, Sonic Dismembrator Model 100, Thermo Fisher Scientific) in 300 µL of lysis buffer ((mmol/L): HEPES, 50; MgCl₂, 1; EDTA, 10, and Triton X, 1%) and centrifugation (1120 g, 4°C/5 min). The supernatant was collected, 10 µL/mL of protease inhibitor cocktail (Roche) was added, and the total protein content was determined using the BCA Protein Assay Kit (Thermo Scientific) and a microplate reader (Multi-Mode Reader, FlexStation 3 Benchtop, Molecular Devices, Sunnyvale, CA, USA). The samples were treated with Laemmli sample buffer (glycerol, 20%; β-mercaptoethanol, 10%; 10% SDS, 40%; Tris, 0.5 mol/L, and pH 6.8, 0.5%; deionized water and bromophenol blue) and heated in a boiling water bath for 3 min (Laemmli 1970).

Forty micrograms of the total protein extracts from pancreatic islets were separated by the 10% SDS–PAGE at 90 V/120 min. The proteins were then transferred from the gel to a nitrocellulose membrane (Trans-Blot Semi-Dry 15-min Electrophoretic Transfer Cell, Bio-Rad) and blocked with 5% of skimmed milk in Tris–HCl, 1 mol/L; NaCl, 5 mol/L, and Tween 20, 0.05% at room temperature for 90 min while shaking. Then, the membrane was incubated overnight at 4°C with rabbit anti-M₃AChR polyclonal primary antibodies 1:1000 (Sigma-Aldrich) followed by peroxidase-conjugated anti-rabbit antibody 1:5000 diluted in Tris–HCl, 20 mmol/L; NaCl, 137 mmol/L, and Tween 20, 0.05%.

Immunoreactive proteins were visualized with ECL (GE Healthcare) and an imager (ImageQuant LAS 500, GE Healthcare). The bands were quantified by densitometry using ImageJ 1.4 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). β-Actin protein content (Santa Cruz Biotechnology; diluted 1:1000 in Tris buffered saline) was utilized for normalization. Representative western blot images were originated from the same membrane.

Electrical activity of vagus nervous

MSG rats that were not vagotomized, either ADX or sham, were anesthetized with thiopental (45 mg/kg BW i.p.) after a 12-h fast. A longitudinal surgical incision was made on the anterior cervical region. Under a dissection microscope, the nerve bundle of the left vagus superior branch was severed from the carotid artery close to the trachea. The nerve trunk was pulled with a fine cotton suture line,

and a pair of recording silver electrodes (0.6 mm in diameter) was placed under the nerve. The nerve was covered with silicone oil to prevent dehydration. The electrode was connected to an electronic device (Bio-Amplificator, Insight, Ribeirão Preto, SP, Brazil) that amplified the electrical signal up to 10,000 times. To exclude the low and high frequencies, recordings from 1 to 80 kHz were filtered. The neural signal output was acquired by an Insight interface (Insight, Ribeirão Preto, SP, Brazil), viewed online and stored by a personal computer running software developed by Insight.

During data acquisition, the animals remained in a Faraday cage to avoid any electromagnetic noise. The nerve activity was analyzed by the number of spikes over the course of 5 s. The spikes were characterized by a depolarization that surpassed 0 mV. After a stabilization of the signal over the course of 2 min, 20 record frames of 15 s were randomly chosen from each animal for spike counting. The average number of spikes was used to calculate the rate of nerve firing for each rat (Gomes *et al.* 2013).

Plasmatic parameters

Immediately after the killing the overnight fasted rats from all groups, blood samples were processed to obtain plasma, which was stored in -80°C until determination of the corticosterone concentration using an RIA kit (ICN Biomedicals, Inc., Aurora, OH, USA). The assay sensitivity was 50 ng/mL, and intra-assay variation was 7%. The leptin levels were determined using an ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). The intra- and inter-assay coefficients of variation were 5.9 and 7.2%, respectively, for leptin.

The glucose concentration was measured by the glucose oxidase method (Trinder 1969) using a commercial kit (Gold Analisa Diagnóstica, Belo Horizonte, MG, Brazil).

The insulin resistance index (IRI) was calculated by the product of fasting insulin ($\mu\text{IU/mL}$) $\times$ fasting glucose (mmol/L) (Fagundes *et al.* 2009, Lima Nda *et al.* 2011, Li *et al.* 2012).

Insulin level analyses

The insulin level from the plasma and pancreatic islet incubation samples was determined by RIA (Scott *et al.* 1981). Human insulin was used as a standard along with an antirat insulin antibody (Sigma-Aldrich) and ^{125}I -labeled recombinant human insulin (PerkinElmer). The intra- and inter-assay coefficients of variation were

12.2 and 9.8%, respectively, for insulin. The detection limit for the insulin level was 1.033 pmol/L. A gamma counter (Wizard² Automatic Gamma Counter, TM-2470, PerkinElmer) was used to measure the radioactivity.

Unless cited, compounds and drugs used in this study were purchased from Sigma-Aldrich.

Statistical analysis

Results are shown as mean $\pm$ s.e.m. Data were submitted to Student's *t*-test or one-way ANOVA, followed by Tukey's post-test analysis, when appropriate, comparing experimental and control groups. *P* values <0.05 were considered to be statistically significant. Tests were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software).

Results

Neonatal treatment with MSG induced many hallmarks of the metabolic syndrome. MSG rats presented less BW, body length, and food intake, 24%; 14% ($P<0.01$) and 17% ($P<0.0001$), respectively, compared with control animals (Table 1). MSG rats also presented a 5% increase in the Lee index ($P<0.05$) and a 1.3-fold increase in visceral fat pad mass ($P<0.0001$). Although normoglycemic, MSG rats presented a two-fold increase in insulin levels ($P<0.0001$) compared with control rats. The leptin and corticosterone levels increased by 14.5-fold and 3-fold ($P<0.001$), respectively. In the peripheral tissue, K_{itt} was reduced by 76% in MSG rats ($P<0.0001$).

Although 100-day-old sham rats gained 3 g compared with their BW at 90 days of age ($P<0.05$), animals

Table 1 Effect of neonatal MSG treatment on biometric and biochemical parameters in adult rats.

Parameters	Control	MSG
Body weight (g)	371.0 $\pm$ 6.5	284.1 $\pm$ 7.2**
Body length (cm)	22.4 $\pm$ 0.3	19.3 $\pm$ 0.3**
Lee index	31.5 $\pm$ 0.3	33.4 $\pm$ 0.4*
Food intake (g/100 g BW)	7.48 $\pm$ 0.09	6.21 $\pm$ 0.15***
Visceral fat pad (g/100 g BW)	0.83 $\pm$ 0.05	1.99 $\pm$ 0.10***
Fasting glucose (mg/dL)	101.9 $\pm$ 1.95	106.3 $\pm$ 3.48 ^{NS}
Fasting insulin (ng/mL)	0.51 $\pm$ 0.02	1.06 $\pm$ 0.07***
Fasting corticosterone (ng/mL)	275.5 $\pm$ 81.7	612.0 $\pm$ 74.1**
Fasting leptin (ng/mL)	1.57 $\pm$ 0.70	22.80 $\pm$ 0.59**
K_{itt} (%/min)	2.99 $\pm$ 0.17	0.71 $\pm$ 0.21***

The data represent the mean $\pm$ s.e.m. obtained from 12 rats per experimental group. The symbols represent significant differences by Student's *t*-test, * $P<0.05$, ** $P<0.01$, *** $P<0.0001$, NS, not significant.

Figure 1

Effect of adrenalectomy, vagotomy, or both surgeries on body weight (A) and food intake (B) in control and MSG rats, 10 days after the surgery. The values are the mean $\pm$ S.E.M. ($n=8-12$ per group) by one-way ANOVA. *** $P<0.0001$ significantly different from the sham, ### $P<0.0001$ significantly different from the ADX-VAG.

that underwent the surgeries, adrenalectomy, vagotomy, and both, displayed an approximately 18 g reduction of BW compared with sham rats ($P<0.0001$), without any significant difference among the operated animals, as shown in Fig. 1A. Adrenalectomy induced a 69% reduction in food intake ($P<0.0001$), and vagotomy caused a 96% ($P<0.0001$) decrease alone and when associated with adrenalectomy compared with sham rats (Fig. 1B).

The control operated animals displayed the same features of the MSG operated rats in both BW gain and food intake, as shown in insets in Fig. 1A and B.

The fasting glucose blood levels are shown in Fig. 2A. Compared with the values of fasting glycemia observed in sham rats, adrenalectomy caused a 25% decrease ($P<0.0001$), whereas vagotomy provoked a 26% increase ($P<0.001$); however, adrenalectomy associated with vagotomy caused a 19% decrease in fasting glycemia ($P<0.001$) compared with sham rats.

As shown in Fig. 2B, fasting insulinemia was reduced by 88% after adrenalectomy ($P<0.0001$) and by 84% ($P<0.0001$) in both operations compared with sham rats. Vagotomy did not reduce insulinemia compared with sham rats. The IRI was decreased 92% by adrenalectomy ($P<0.0001$) and by 86% ($P<0.0001$) in ADX-VAG, compared with sham rats (Fig. 2C).

Although glycemia was unaltered during ivGTT among the MSG groups (Fig. 3A), the insulin level was reduced by 21% ($P<0.001$) upon adrenalectomy, 46% upon vagotomy ($P<0.001$), and 53% upon both surgeries ($P<0.0001$) (Fig. 3B).

Vagotomized rats showed 20% less corticosterone than sham rats, but the difference was not statistically significant (Fig. 4). The levels of corticosterone in ADX and ADX-VAG rats were undetectable. Electrical vagus records are shown in Fig. 5; adrenalectomy provoked a reduction of 41% in vagus nervous activity ($P<0.0001$) compared with sham rats.

In general, isolated pancreatic islets from sham rats displayed a curved dose-response to glucose, as shown in Fig. 6A. In addition, pancreatic islets from adrenalectomized and vagotomized rats increased their insulin release with an increase in the glucose concentration; however, the operations did not present the same profiles, with an increase only in the highest glucose concentration. In the same way, adrenalectomized rats showed a lower insulin release in comparison to sham-operated animals only in the highest glucose concentration. The other operated animals secreted less insulin than sham-operated rats when stimulated by different glucose concentrations.

Figure 2

Effect of adrenalectomy, vagotomy, or both surgeries on the fasting glycemia (A), insulinemia (B), and IRI (C) in MSG rats, 10 days after the surgery. The values are the mean $\pm$ S.E.M. ($n=8-12$ per group) by one-way ANOVA. ** $P<0.001$ and *** $P<0.0001$ significantly different from the sham; ### $P<0.0001$ significantly different from the ADX-VAG.

Figure 3

Effect of adrenalectomy, vagotomy, or both surgeries on the glycemia (A) and insulinemia (B) during ivGTT in MSG rats, 10 days after the surgery. The insets in each figure represent the respective areas-under-the-curve (AUC) of glycemia and insulinemia. Values are the mean $\pm$ s.e.m. ($n=8-12$ per group) by one-way ANOVA. ** $P<0.001$ and *** $P<0.0001$ significantly different from the sham; ## $P<0.001$ significantly different from ADX-VAG.

In the basal glucose concentration (5.6 mmol/L), the selective M_3 AChR agonist (oxotremorine-M) potentiates insulin secretion from sham, adrenalectomized rats (approximately 80%; $P<0.01$), vagotomized rats, and adrenalectomized and vagotomized rats (50%, $P<0.01$), as shown in Fig. 6B. At the high glucose concentrations (16.7 mmol/L), the potentiation of glucose-induced insulin secretion by oxotremorine-M increased two-fold compared with insulin secretion induced by glucose only in sham rats, whereas this insulinotropic effect of oxotremorine-M increased four-fold in adrenalectomized as well as vagotomized rats ($P<0.001$). No action of oxotremorine-M in the pancreatic islets from rats that were adrenalectomized and vagotomized was observed (Fig. 6B).

Protein expression of M_3 AChR from isolated pancreatic islets is shown in Fig. 7. Vagotomy did not cause changes in M_3 AChR expression compared with sham

Figure 4

Effect of adrenalectomy, vagotomy, or both surgeries on the corticosterone blood levels from the MSG rats, 10 days after the surgery. The values are the mean $\pm$ s.e.m. ($n=8$ per group) by one-way ANOVA. *** $P<0.0001$ significantly different from the sham; ### $P<0.001$ significantly different from ADX-VAG.

rats. Adrenalectomy and the combined surgeries caused a decrease in M_3 AChR expression (26 and 46%, respectively, $P<0.05$).

Discussion

Data from this study demonstrate that disruption of insulin secretion control in hypothalamic obesity is associated with impairment of the HPA axis and PNS, and each system contributes individually or in combination. The onset of obesity is associated with low sympathetic and high parasympathetic activities to sustain the accumulation of energy sources, such as fat tissue, decreasing catabolism, and increasing anabolism. Regarding the BW of MSG rats, we observed that they were lighter than respective control animals, which was the characteristic phenotype of this model as described by Remke *et al.* (1988), Dolnikoff *et al.* (2001), and Schoelch *et al.* (2002). Although there were no overweight rats, the MSG-obese rats were found to have more fat pad stores as compared with control animals (Bernardis & Patterson 1968, Bray & York 1998), which was correlated with lower growth hormone (Bakke *et al.* 1978, Acs *et al.* 1982) and reduced basal metabolic rate (Arndt *et al.* 1991).

Vagotomy attenuates obesity from different origins (Bray & York 1998). In this study, subdiaphragmatic VAG was able to reduce BW gain. Decreasing corticosterone levels by adrenalectomy in obese animals also attenuates

Figure 5

Electrical activity of the vagus nerve from MSG-sham and ADX rats, 10 days after the surgery. Representative records of nerve discharges for each experimental group are shown below the bars. The values are the mean $\pm$ S.E.M. ($n=8$ per group) by Student's t -test, **** $P<0.001$.

obesity (Dallman *et al.* 2007). Nevertheless, patients with Cushing syndrome submitted to adrenalectomy also showed a reduction of obesity (Chow *et al.* 2008). In this study, MSG-obese rats submitted to adrenalectomy presented low corticosterone levels and attenuated obesity. Vagotomy or adrenalectomy reduced chow consumption in MSG rats; however, our findings suggest that interruption of vagus nervous activity plays a crucial role in food behavior compared with adrenal cortical activity. These data corroborate other previous studies in obese patients and animal obese models, which had reduced food intake when submitted to vagotomy (Cox *et al.* 2004, Yin *et al.* 2007, Zheng *et al.* 2009). Vagotomy prolongs gastric emptying and can affect the release of gastrointestinal hormones, such as ghrelin and glucagon-like peptide 1 (GLP1), which regulate orexigenic and anorexigenic hypothalamic neuropeptides. Vagotomy in obese rats decreased orexigenic signals by neuropeptide Y (NPY) in

Figure 6

Effect of adrenalectomy, vagotomy, or both surgeries on the glucose-induced insulin secretion capacity (A) and on the insulinotropic responsiveness of the M_3 AChR (B) in the pancreatic islets from MSG rats, 10 days after the surgery. The values are mean $\pm$ S.E.M. of insulin secreted by the isolated pancreatic islets obtained from six rats of three different litters for each experimental group. The symbols over the bars represent significant differences among the groups by one-way ANOVA, where in Fig. A (*) represents the comparison between each one of the group with sham group at each glucose concentration (* $P<0.01$ and *** $P<0.0001$) and (#) represents the comparison between each one of the group at 8.3 or 16.7 mmol/L glucose concentrations with their counterpart at 5.6 mmol/L (* $P<0.001$). In Fig. B, (*) represents the comparison between each group with the sham group at each glucose and/or Oxo-M treatment (* $P<0.01$ and *** $P<0.0001$), (#) represents the comparison between each group treated with Oxo-M and their counterpart group without Oxo-M at 5.6 mmol/L glucose (* $P<0.001$), and (Ω) represents the comparison between each group treated with Oxo-M and their counterpart group without Oxo-M at 16.7 mmol/L glucose (Ω $P<0.001$). Oxo-M, oxotremorine-M.

the hypothalamus (Asakawa *et al.* 2001). Because MSG animals had increased corticosterone and this hormone increases appetite for palatable food, adrenalectomized animals had a great decrease in food intake. It is expected that after such surgeries, the animals markedly decrease their food intake and lose weight, but it seems that adrenalectomy is less traumatic to decrease food intake than vagotomy. However, without aldosterone, adrenalectomized animals lose more sodium, both via the kidney and gut. Moreover, gut sodium is important as a cotransporter for glucose and amino acid uptake.

Figure 7 Effect of adrenalectomy, vagotomy, or both surgeries on M₃AChR protein expression in the pancreatic islets from six rats of three different litters per group for each experimental group, 10 days after the surgery. The values are the mean $\pm$ S.E.M. by one-way ANOVA. *** $P < 0.0001$ significantly different from the sham; ## $P < 0.001$ and ### $P < 0.0001$ significantly different from ADX-VAG. Representative western blot images were carried out from the same membrane.

The reduction of body mass found in isolated surgeries is the same as that found in animals with both surgeries, showing that there is no additive effect of the combined surgeries. Glucocorticoids are able to increase fat mobilization as well as stimulate lipogenesis (Lee *et al.* 2014). Glucocorticoids in excess were found to decrease SNS activity (Grassi *et al.* 2001). This observation associated with high vagus nervous activity in MSG-obese rats suggests that imbalanced ANS activity contributes to high fat tissue accretion.

Chronic high corticosteroid administration is well known to cause insulin resistance and hyperinsulinemia (Yang *et al.* 2012). An elevated release of insulin is an effort of the pancreatic β -cell to maintain normal blood glucose (Litvinova *et al.* 2015). In this study, adrenalectomy abolished corticosterone serum levels with a parallel decrease in fasting glycemia and insulinemia. As expected, vagotomy provoked hyperglycemia, but surprisingly with no alteration in fasting insulinemia. In humans and many mammals, vagotomy provokes a fall in blood insulin levels. Surgery causes blunting of ACh release in pancreatic β -cell neuronal ends, which reduces the cholinergic response of glucose-induced insulin secretion (GIIS) (Henquin 2000). Glucocorticoids induce gluconeogenesis, which supports high blood glucose levels in humans and rodents submitted to chronic treatment with glucocorticoids (Rafacho *et al.* 2014). High HPA axis activity induces an enhancement of insulin secretion. Cutting of the vagus nerve and the effect on insulin release may be compensated by the chronic

corticosterone-induced insulin secretion. Bernal-Mizrachi and co-workers showed that dexamethasone-treated rats displayed a disruption of the afferent vagus, glucose intolerance, and hypertension and the vagotomy was able to reverse these effects (Bernal-Mizrachi *et al.* 2007). They consider that glucocorticoid-induced glucose intolerance required intact vagal afferent fibers. Indeed, the complete mechanism of glucocorticoids action in stimulated vagus nerve is still not well understood (Bernal-Mizrachi *et al.* 2007). Although, it is important to highlight that intracerebroventricular (ICV) dexamethasone injections in vagotomized rats did not induce increased food intake, BW, or insulin resistance (Cusin *et al.* 2001). This central effect of glucocorticoids appears to be mediated, at least in part, by the PNS (Sainsbury *et al.* 1997).

In feeding conditions, using the ivGTT method, glycemia did not change, although insulin was lower after all surgeries. This result is suggestive of an increase in insulin sensitivity. Our data corroborate a previous report in which vagotomy improved insulin sensitivity in rat pups treated neonatally with MSG (Balbo *et al.* 2007). Importantly, glucocorticoids and vagal hypertonia suppression have different effects depending on fasting and feeding conditions. Decreasing corticosterone by adrenalectomy in fasting conditions appears to promote a normal response of insulin to glucose; however, in feeding conditions adrenalectomy seems to improve insulin sensitivity. This last effect is even more pronounced when vagotomy is performed in feeding conditions, whereas the insulin response was inappropriate in fasting conditions. The HPA axis and PNS show an influence on glycemic homeostasis, and together they may act synergistically on insulin secretion control.

As observed *in vivo*, both systems participate in the control of insulin levels, and isolated pancreatic islet function showed dependence for the two systems. Islets from obese rats presented a higher response to glucose than those from lean rats (Miranda *et al.* 2014). Vagotomy and adrenalectomy or both improved GIIS in isolated islets. Glucose is the main signal to stimulate β -cells to produce and secrete insulin. Herein, we suggest that hypercorticism associated with hyperactivity of vagus nerves contributes to functional changes in pancreatic β -cells in MSG-obese rats. In fact, as previously reported, hyperactivity of vagus nerves reduces apoptosis and increases proliferation in pancreatic β -cells (Medina *et al.* 2013), and excessive corticosteroids induce β -cell proliferation leading to insulin oversecretion (Rafacho *et al.* 2014).

M₃AChR activity might also contribute to the glucose insulinotropic response in β -cells. Lean mice with M₃AChR knockout present low insulin blood levels in fasting or fed

conditions and a low glucose insulintropic response in isolated pancreatic islets (Yamada *et al.* 2001). Nevertheless, pancreatic islets of mice with M₃AChR overexpression show a high glucose insulintropic response (Gautam *et al.* 2006). Indeed, this study shows that the glucose response was diminished in operated MSG rats, including both surgeries. These data can be associated with the decrease of M₃AChR protein expression also observed in operated animals, suggesting a mechanism involving M₃AChR quantity and/or transduction signal changes; however, independent of the mechanism, both systems might modulate β -cell function.

Vagotomy in MSG-obese rats increases the cholinergic insulintropic response (Balbo *et al.* 2002), as was shown with a selective M₃AChR agonist, oxotremorine-M, in this study. The cholinergic response was decreased in pancreatic islets isolated from MSG-obese rats compared with lean rats; however, the protein expression of M₃AChR was increased (Miranda *et al.* 2014). In the animals treated with dexamethasone, an enhancement of M₃AChR in pancreatic islets was found (Angelini *et al.* 2010). In this study, adrenalectomy significantly reduced the protein expression of M₃AChR in the islets, whereas vagotomy alone did not show a statistical change. When adrenalectomy was combined with vagotomy, the decrease in M₃AChR protein expression was even higher. Thus, together the HPA axis and ANS play an important role in regulating insulin secretion through M₃AChR.

For the first time, we have demonstrated that upon reducing corticosterone blood levels by adrenalectomy in MSG-obese rats, glucose homeostasis was improved as was pancreatic function by inhibiting insulin oversecretion. High protein expression of M₃AChR was recently registered (Miranda *et al.* 2014) as was high vagus nerve activity in the MSG-obesity model reported by our group (Balbo *et al.* 2002, Miranda *et al.* 2014). Adrenalectomy as observed in this study was able to reduce M₃AChR levels in the islets and inhibit electrical vagal activity. These results indicate that enhanced activity of the HPA axis augments vagal activity, which also allows changes in the protein expression and function of M₃AChR from pancreatic β -cells.

Both systems, the HPA axis and ANS, influence the GIIS process, which can include changes in M₃AChR. The results also provide evidence to reinforce that pancreatic β -cell M₃AChR might be a new therapeutic target for metabolic diseases.

Taken together, data in this study suggest a synergistic action of the HPA axis and PNS hyperactivity on pancreatic β -cell physiology in MSG-obese rats leading to

insulin oversecretion, which allows these animals to develop obesity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R A M, R T, and P C F M were responsible for the conception and design of the study. R T, R A M, J C O, L F B, and C C S F collected, analyzed, and interpreted the data. R A M, P C L, P C F M, and E G M drafted and/or critically reviewed the manuscript. All authors approved the final version of the manuscript, all persons designated as authors qualify for authorship, and all those who qualify for authorship have been listed.

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