Insulin Oversecretion in MSG-Obese Rats is Related to Alterations in Cholinergic Muscarinic Receptor Subtypes in Pancreatic Islets

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Key Words
MSG-obese animals • Muscarinic receptor subtypes • Insulin secretion

Abstract

Background/ Aims: Impaired pancreatic beta cell function and insulin secretion/action are a link between obesity and type 2 diabetes, which are worldwide public health burdens. We aimed to characterize the muscarinic acetylcholine receptor (mAChR) M\textsubscript{1}–M\textsubscript{4} subtypes in isolated pancreatic islets from pre-diabetic obese rats that had been treated neonatally with monosodium L-glutamate (MSG). Methods: At 90 days of age, both the MSG and the control groups underwent biometric and biochemical evaluation. Anti-muscarinic drugs were used to study mAChR function either \textit{in vivo} or \textit{in vitro}. Results: The results demonstrated that atropine treatment reduced insulin secretion in the MSG-treated and control groups, whereas treatment with an M\textsubscript{2} mAChR-selective antagonist increased secretion. Moreover, the insulinostatic effect of an M\textsubscript{3} mAChR-selective antagonist was significantly higher in the MSG-treated group. M\textsubscript{1} mAChR and M\textsubscript{3} mAChR expression was increased in the MSG-obese group by 55% and 73%, respectively. In contrast, M\textsubscript{2} mAChR expression decreased by 25% in the MSG group, whereas M\textsubscript{4} mAChR expression was unchanged. Conclusions: Functional changes in and altered content of the mAChR (M\textsubscript{1}–M\textsubscript{4}) subtypes are pivotal to the demand for high pancreatic beta cell insulin secretion in MSG-obese rats, which is directly associated with vagal hyperactivity and peripheral insulin resistance.
Introduction

Obesity is a worldwide problem that is associated with cardiovascular, respiratory and gastrointestinal dysfunction, dyslipidemia, insulin resistance, and type 2 diabetes, among other metabolic syndrome components [1-3].

One feature of obesity in both animals and humans is a breakdown in the balance of autonomic nervous system (ANS) activity because of low sympathetic nervous system (SNS) activity and a parallel increase in parasympathetic nervous system (PNS) activity. Imbalance of the ANS induces hormonal and metabolic changes favoring the development of obesity [4, 5]. Among PNS actions, it is important to highlight the potentiating effect of insulin secretion on the endocrine pancreas. Vagal activation of the pancreas promotes acetylcholine (ACh) release, which triggers signaling events via muscarinic acetylcholine receptor (mAChR) activation, which culminates in glucose-stimulated insulin secretion [6, 7].

In total, five different mAChR subtypes (M₁–M₅) have been characterized and are widely distributed in most cells. However, in pancreatic islets, four mAChR subtypes (M₁–M₄) have been identified [8-11]. Pancreatic mAChR subtypes are involved in fine regulation and adjustment of insulin secretion. The mAChR subtypes found in these cells are divided into insulinotropic receptors (M₁ and M₃), which potentiate glucose-induced insulin secretion (GIIS), and insulinostatic receptors (M₂ and M₄), which attenuate the cholinergic effect.

The insulinotropic ACh response in pancreatic islets is mostly generated by M₃ mAChR and to a lesser degree by M₁ mAChR [10, 12]. These receptors are coupled to G protein subtype Gq/11 [13], which activates phospholipase C (PLC), thus cleaving phosphatidylinositol-4, 5-bisphosphate (PIP) into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP₃). These products are responsible for the subsequent mobilization of intracellular calcium, resulting in insulin granule exocytosis by beta cells [14]. In contrast, M₂ mAChR and M₄ mAChR are coupled to inhibitory G protein subtype Gi/o [15], which inhibits adenylate cyclase (AC) activity. This inhibition reduces intracellular cAMP levels, contributing to the decreased GIIS that is potentiated by the odd mAChR. The PNS-mediated GIIS modulation greatly depends on mAChR subtype composition.

Regarding obese humans and animals, increased parasympathetic activity and hyperinsulinemia [16-18] are associated with insulin oversecretion [19], which supports the idea that mAChR composition in pancreatic islets may be compromised in these individuals. Thus, the use of an experimental model that presents these features is essential for mAChR characterization in the pancreatic beta cells of obese individuals.

In fact, an experimental model that is already well studied and that reconciles increased parasympathetic activity, hyperinsulinemia and obesity among other components of the metabolic syndrome is the pre-diabetic obese rat model induced by neonatal monosodium L-glutamate (MSG) administration [20-22]. At excessive concentrations, this amino acid/neurotransmitter has a neurotoxic effect that damages cells in the hypothalamic arcuate nucleus (ARC) and adjacent areas. ARC neurons are crucial for the regulation of metabolic homeostasis, which includes insulin secretion and insulin tissue action [23].

The goal of the present study was to determine whether insulin oversecretion in pre-diabetic MSG-obese rats is associated with disarrangement in M₁-M₄ mAChR composition and activity on the pancreatic beta cell cholinergic response.

Materials and Methods

Animals

All of the animal protocols were approved by the State University of Maringá Animal Ethics Committee. Neonatal male rats were subcutaneously injected during the first 5 days of life with 4 mg MSG/g body weight (bw). Control animals received saline solution (NaCl, 0.9% w/v). Both of the animal groups were weaned on the 21st day of life. All of the animals were housed under controlled conditions in a 12-h light-
dark cycle (lights on from 7 a.m. to 7 p.m.) and at a temperature of 21 ± 2 °C. Water and a standard rodent chow diet (Nuvital®, Curitiba, PR, Brazil) were offered ad libitum.

Obesity was assessed based on bw and nasoanal length, which was used to calculate the rodent body mass index or Lee index [24]. Retroperitoneal, epididymal and visceral fat pads were removed and weighed.

**Intravenous glucose tolerance test (IVGTT) and the effects of muscarinic agonists and antagonists in vivo**

At 90 days of age, MSG and control rats underwent surgery consisting of cannula implantation into the right jugular vein as has been previously described [25]. A glucose load (1 g/kg bw) was infused, and blood samples (350-400 µl) were collected immediately before the glucose load (0 min) and at 5, 15, 30 and 45 min after glucose administration (n=16-18).

To study the influence of the muscarinic system on glycemic changes, 5 min prior to the glucose load, we intraperitoneally injected Ach as a physiological agonist (27 nmol/l/kg bw) or atropine as a nonselective antagonist (Atr; 20 nmol/l/kg bw) into another batch of animals from each group (n=12-16). To investigate whether the increased insulin response to glucose as observed in this model is related to M₃mAChR function, we studied the effects of a selective M₃mAChR antagonist (4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; 0.21 µmol/l/kg bw) on both experimental groups (n=14-16). Blood samples were taken at the times indicated above, and the obtained plasma samples were stored at -20 °C for further analyses. The glucose concentration was determined by the glucose oxidase method [26] using a commercial kit (Gold Analisa®, Belo Horizonte, MG, Brazil). The insulin levels were determined using a radioimmunoassay [27] with a gamma counter (Wizard² Automatic Gamma Counter, TM-2470, PerkinElmer®, Shelton, CT, USA). For the radioimmunoassay, we used human insulin as a standard, anti-rat insulin antibodies (Sigma-Aldrich®, St. Louis, MO, USA) and ¹²⁵I-labelled recombinant human insulin (PerkinElmer®, Shelton, CT, USA).

The intra- and interassay coefficients of variation for insulin were 12.2% and 9.8%, respectively. The detection limit for insulin was 1.033 pmol/l.

Corticosteronemia and leptinemia were determined using a radioimmunoassay commercial kit (MP Biomedicals®, Eschwege, Germany) and an ELISA kit (Enzo® Life Sciences, Plymouth Meeting/PA, USA), respectively.

**Insulin sensitivity**

Another batch of rats from both control and MSG groups underwent cannula implantation. After a 6-h fast and without anesthesia the rats were submitted to an intraperitoneal insulin tolerance test (ipITT; 1 U insulin /kg bw). Samples for blood glucose measurements were collected at zero (basal), 5, 15, 30 and 45 min after insulin injection as described for the IVGTT. The glucose tissue uptake rate or the plasma glucose disappearance rate constant (Kₐ) was calculated using the formula 0.693 / (t₁/₂), as indicated in a previous report [28]. Plasma glucose t₁/₂ was calculated from the slope of the least square analysis of the plasma glucose concentrations during the linear phase of decline.

**Pancreatic islet isolation and stimulation of insulin secretion in vitro**

Pancreatic islets were isolated by the collagenase technique [29] with certain adaptations, as previously described by our group [30]. To adjust the isolated islets to a baseline glucose concentration (5.6 mmol/l), four islets per well were pre-incubated for 60 min in 1 ml of Krebs-Ringer solution (in mmol/l: NaCl, 115; NaHCO₃, 24; KCl, 1.6; MgCl₂·6H₂O, 1; CaCl₂·2H₂O, 1; and BSA, 15) at pH 7.4 containing 5.6 mmol/l glucose. This solution was gassed with 95% O₂ mixed with 5% CO₂ to maintain pH 7.4. To study mAChR function, the islets were incubated for an additional 60 min in Krebs-Ringer solution containing 8.3 mmol/l glucose or 8.3 mmol/l glucose plus 10 µmol/l Ach in the presence of neostigmine (10 µmol/l). To block M₃mAChR and M₅mAChR subtype function, the selective M₃mAChR antagonist methoctramine (MTT; 1 µmol/l) and 4-DAMP (100 µmol/l) were used, respectively. At least three rats from three different litters were used for each experimental procedure per group.

All of the drugs described above for studying mAChR function were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Western blotting**

M₃mAChR, M₅mAChR, M₃mAChR and M₅mAChR protein content in isolated pancreatic islets from the 90-day-old rats was determined by immunoblotting. In total, 300 islets from each experimental group were
frozen at -80 °C and subjected to posterior sonication (Sonic Dismembrator Model 100, Fischer Scientific, Waltham, MA, USA) in lysis buffer (in mmol/l: HEPES, 50; MgCl₂, 1; and EDTA, 10; plus Triton X, 1%, v/v) containing a protease inhibitor cocktail (Roche®). The islets were then centrifuged at 12,000 rpm for 5 min at 4 °C. Total protein content was determined using a BCA™ Protein Assay Kit (Thermo Scientific®, Rockford, IL, USA) and a microplate reader (Multi-Mode Reader, FlexStation® 3 Benchtop, Molecular Devices, Sunnyvale, CA, USA). The samples were treated with Laemmli sample buffer (w/v: glycerol, 20%; β-mercaptoethanol, 10%; 10% sodium dodecyl sulfate (SDS), 40%; and 0.5 mol/l Tris, pH 6.8, 0.5%; plus deionized water and bromophenol blue) [31].

Total protein extracts (60 µg) from the pancreatic islets were separated by 10% SDS-PAGE at 90 V for 120 min. The proteins were then transferred from the gel to a nitrocellulose membrane by the Trans-Blot® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad®, Hercules, CA, USA) and blocked with 5% skim milk in Tween-Tris-buffered saline (TTBS; Tris-HCl, 1 mol/l; NaCl, 5 mol/l; and Tween 20, 0.05%, v/v) for 90 min with continuous shaking. Membranes were incubated overnight at 4 °C with rabbit anti-M₁ mAChR, anti-M₂ mAChR and anti-M₃ mAChR (Sigma-Aldrich®, St. Louis, MO, USA) and rabbit anti-M₄ mAChR (Santa Cruz Biotechnology®; Santa Cruz, CA, USA) polyclonal primary antibodies at a 1:1000 dilution, followed by incubation with peroxidase-conjugated anti-rabbit antibodies at a 1:5000 dilution (Sigma-Aldrich®, St. Louis, MO, USA). Antibodies were diluted in buffer (20 mmol/l Tris-HCl, 137 mmol/l NaCl and 0.05% Tween 20). Immunoreactive proteins were visualized with ECL (GE Healthcare, Buckingham, Shire, UK) and a scanner (Amersham Storm™ 860 Imaging System, Gene Tool, Milpitas, CA, USA). The bands were quantified by densitometry using Image J 1.4 software (Wayne Rasband, National Institutes of Health, Bethesda, MA, USA).

β-actin protein content (Santa Cruz Biotechnology®, Santa Cruz, CA, USA; diluted 1:1000 in TTBS) was utilized for normalization.

**Statistical analyses**

The data are given as the means ± SEM and were subjected to Student’s t test. A value of P <0.05 was considered to be statistically significant using GraphPad Prism version 6.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

**Results**

**Effect of MSG treatment in rats: biometric and biochemical parameters**

Several studies demonstrate that neonatal MSG treatment induced obesity in rats [32-34]. To confirm that neonatal MSG treatment was effective in inducing obesity, we analyzed biometric parameters including bw, the Lee index and main fat stores as demonstrated in Table 1. The MSG-obese rats demonstrated a 66%, 80% and 134% increase in the retroperitoneal, epididymal and visceral fat pads, respectively compared with control animals (Table 1).
As demonstrated in Table 1, the biochemical parameters of corticosteronemia and leptinemia were 2- and 11-fold higher, respectively, in the MSG group than in the control group (\( P < 0.01 \)). Although normoglycemic, rats from the MSG group demonstrated fasting hyperinsulinemia (114%, \( P < 0.0001 \)) and their HOMA-IR was increased by 103% (\( P < 0.001 \)) compared with the control rats (Table 1).

An important feature of metabolic syndrome is a glucose homeostasis disorder that is triggered by hyperinsulinemia and insulin resistance [35, 36]. Results from the present work demonstrate that MSG-obese rats displayed glucose intolerance, with a 25% increase in the AUC during the IVGTT (\( P < 0.05 \)), as shown in the insert in Fig. 1a. As observed in Fig. 1b, the insulinemia AUC during the IVGTT increased by 261% (\( P < 0.0001 \)) in the MSG-obese rats compared with the control group.

To determine insulin sensitivity, we performed an ipITT. Neonatal MSG treatment caused a 4-fold decrease in \( K_{itt} \) (\( P < 0.0001 \)), indicating that these animals are insulin resistant (Fig. 2).

**Effect of treatment (in vivo) with mAChR agonists**

During the IVGTT, we confirmed the involvement of mAChR on insulin secretion by blocking Atr-induced insulin secretion, which displayed greater inhibition in the MSG-obese animals. Likewise, \( M_3 \)AChR blockade demonstrates greater inhibition, suggesting that the \( M_3 \)AChR subtype is more sensitive in pancreatic beta cells from MSG-obese rats. Figure 3a
demonstrates the results of *in vivo* mAChR agonist treatment. ACh treatment increased the AUC of the insulinemia increment percentage during the IVGTT in control rats by 53% (*P* < 0.05), whereas this effect was not observed in the MSG-obese rats. In contrast, Atr treatment decreased insulinemia in both the control (81%) and the MSG (91%, *P* < 0.0001) groups; likewise, 4-DAMP treatment decreased insulinemia by 55% in the control rats (*P* < 0.0001) and 91% (*P* < 0.0001) in the MSG-obese rats compared with the animals without 4-DAMP treatment (Fig. 3b).
Effect on GIIS in isolated pancreatic islets from MSG-obese rats (in vitro treatment)

To evaluate the function of the major mAChR subtypes (M$_2$ and M$_3$) involved in modulating insulin secretion, islets were incubated with antimuscarinic drugs. As expected (Fig. 4a), the GIIS in the islets isolated from MSG-obese rats was approximately 2-fold higher than in control animals (87.53 ± 3.75 pmol/l, control; 169.3 ± 10.06 pmol/l, MSG; $P < 0.0001$). Conversely, the insulinotropic effect of ACh was more than 2-fold lower in the islets from MSG-obese rats compared with control animals (303.7 ± 13.52 pmol/l, control; 262.6 ± 10.74 pmol/l, MSG; $P < 0.0001$).

Treatment with the nonselective mAChR antagonist Atr reduced insulin secretion in both groups. The insulinostatic effect of Atr was 73% in the control group and 52% in the MSG group relative to Ach-potentiated GIIS in each group (Fig. 4b, $P < 0.0001$).

Insulin secretion was increased by the M$_2$ mAChR-selective antagonist MTT in both groups. Whereas the magnitude of this increase was 154% in MSG-obese rat islets, secretion was increased by 32% in the control group ($P < 0.0001$). In contrast, the insulinostatic effect of 4-DAMP, an M$_3$ mAChR-selective antagonist, was observed in both groups and was significantly higher in the MSG group (53%, $P < 0.001$) than in the control group (41%, $P < 0.0001$) compared with the Ach-potentiated GIIS in each group.

**MSG treatment alters cholinergic mAChR subtypes in pancreatic islets**

Experimental studies have determined that in the pancreas, paired mAChR subtypes exert inhibit Ach-mediated GIIS [25], while the odd mAChR subtypes are responsible for the
insulinotropic effect [9, 37], suggesting that the Ach-mediated insulin secretion enhancement may depend on pancreatic beta-cell membrane mAChR composition. We discovered altered M<sub>1</sub>-M<sub>4</sub>mAChR subtype expression in isolated pancreatic islets by western blotting analyses. Neonatal MSG treatment increased M<sub>1</sub>mAChR and M<sub>3</sub>mAChR subtype protein expression in isolated pancreatic islets by 55% and 73%, respectively, compared with the control group (Figs 5a, c; P < 0.001). In contrast, M<sub>2</sub>mAChR expression decreased by 25% in MSG-obese rat islets compared with control group islets (Fig. 5b, P < 0.001). No difference in M<sub>4</sub>mAChR expression was observed (Fig. 5d).

Discussion and Conclusions

In the current study, we characterize the protein expression of the four mAChR subtypes (M<sub>1</sub>-M<sub>4</sub>) in rat pancreatic islets from both control and MSG-obese rats for the first time. mRNA expression of the main insulinotropic (M<sub>1</sub> and M<sub>3</sub>) mAChR subtypes has been characterized in the RINm5F and INS-1 insulin-producing cells, and even in isolated pancreatic islets from control rats [9], but only the M<sub>1</sub> and M<sub>3</sub> subtypes were examined. Similarly, our group previously reported that the four mAChR subtypes are present in the BRIN BD11 clonal pancreatic beta cell line [38]. However, complete characterization of the four mAChR subtypes has not yet been described in pancreatic beta cells or even Langerhans' islets from rodents or humans.

Interestingly, our data demonstrate that the ratio of insulinotropic to insulinostatic mAChR subtype protein expression ((M<sub>1</sub> plus M<sub>3</sub>)/(M<sub>2</sub> plus M<sub>4</sub>)) in pancreatic islets from control rats was near one. The cholinergic response produced a slightly higher effect on M<sub>3</sub>mAChR than on the M<sub>2</sub>mAChR subtype, which are the main opposing mAChR subtypes (M<sub>2</sub> and M<sub>3</sub>) on insulin secretion as assessed by selective antagonist blockage. The major physiological role of ACh in insulin secretion is indicated by M<sub>3</sub>mAChR responsiveness [7, 8]. Studies examining mAChR activity used selective pharmacological antagonists to characterize M<sub>1</sub>, M<sub>2</sub> and M<sub>3</sub> subtype function and binding in rat pancreatic islets [39]. Beyond functional study, it is remarkable that in the present work, we demonstrate protein expression of the four (M<sub>1</sub>-M<sub>4</sub>) mAChR subtypes not only in control rat pancreatic islets but also in islets from rats with metabolic dysfunction.

In the current study, we confirm previous reports of impaired metabolic features that are well characterized in MSG-obese rats that display peripheral insulin resistance, which is associated with high GIIS and high vagus nerve activity. Similarly, although they display a large glucose insulinotropic effect, MSG-obese rats have a weak cholinergic response [20, 25, 33].

Despite a weak cholinergic response to GIIS in isolated islets, the M<sub>3</sub>mAChR-selective antagonist more profoundly blocked insulin secretion, which was associated with high vagal activity in MSG-obese rats and may be an important promoter of hyperinsulinemia in those rats. In contrast, there was substantial functional response of the M<sub>3</sub>mAChR subtype under selective antagonist action in MSG-obese rats both in vivo and in vitro. This response may be a compensatory mechanism for the insulin oversecretion that is observed in MSG-obese rats, which would inhibit more insulin secretion than what is already present. Although the ratio of functional M<sub>3</sub>mAChR to M<sub>3</sub>mAChR cholinergic responses was close to one, which affected insulin secretion in control rat islets, this ratio more than doubled in MSG-obese rats.

Here, we report high M<sub>3</sub>mAChR and M<sub>3</sub>mAChR subtype and low M<sub>3</sub>mAChR protein expression in pancreatic islets from MSG-obese rats for the first time. Consistent with the functional study, these data suggest that altered mAChR composition could be shaped in MSG-obese rat pancreatic islets to help the rats meet the insulin secretion demand resulting from peripheral insulin resistance, which can contribute to pancreatic beta cell exhaustion.

Nearly equivalent M<sub>3</sub>mAChR and M<sub>3</sub>mAChR mRNA expression in rat pancreatic islets has been reported [9]. Similarly, our data demonstrate that pancreatic islets from either
control or MSG-obese rats display equivalent M₃mAChR and M₁mAChR protein expression. However, the ratio of insulinotropic and insulinostatic [(M₁ plus M₄)/(M₂ plus M₃)] mAChR subtypes was 2-fold higher in MSG-obese rats relative to control rats. It is important to note that we did not study the M₅mAChR subtype because no extensive expression of this subtype has been demonstrated in pancreatic islets or in other clonal insulin-producing cells [9, 38].

Obesity is strongly associated with insulin resistance and type 2 diabetes, which have been associated with imbalanced ANS activity, i.e., low sympathetic and high parasympathetic pathway activity [40, 41]. However, the mechanisms underlying cholinergic modulation are debated. The high corticosteronemia that is observed in MSG-obese rats may also change mAChR composition in their pancreatic islets. Several lines of evidence have indicated an association between glucocorticoid action and insulin resistance including insulin oversecretion. Generally, glucocorticoids are potent modulators of gene transcription [42]. Chronic treatment with dexamethasone, a synthetic glucocorticoid, provokes insulin resistance and insulin secretion and increases M₃mAChR protein expression in rat pancreatic islets [43]. Although dexamethasone-treated rats represent a different model of insulin oversecretion, MSG-obese rats are also insulin resistant, have islets that oversecrete insulin and display high M₃mAChR protein expression, similar to islets from dexamethasone-treated rats [43]. However, further studies should be performed to better understand glucocorticoid involvement in the cholinergic pathway impairment that occurs in MSG-obese rats. Mechanisms other than glucocorticoid action have also been proposed to influence the insulin secretion machinery, such as incretins [44], adrenergic-stimulated pathways [45] and autocrine insulin action [46]; changes in one of these pathways could certainly modulate mAChR composition.

As previously demonstrated, genetically modified mice lacking the M₃mAChR subtype in pancreatic beta cells displayed weak insulin secretion during a cholinergic response [47]. This finding is closely correlated with our work because we present high M₃mAChR expression, which is associated with increased insulin levels. In an opposite model, transgenic mice overexpressing M₃mAChR in pancreatic beta cells display metabolic syndrome resistance [48]. Similarly, in another study, it was demonstrated that mutant mice overexpressing the constitutively active Q490L mutant protein, which encodes a persistently active M₃mAChR subtype that constantly potentiates GIIS in pancreatic β-cells, are resistant to high fat diet-induced obesity [49]. These data support the suggestion that glucose- and insulin-coupled processes have a close link to the cholinergic response pathway, which may induce mAChR subtype composition changes in beta cells and interfere in the glucose metabolism pathways that stimulate insulin release.

High-carbohydrate diet-induced metabolic syndrome causes high expression of M₃mAChR mRNA in pancreatic islets in addition to proteins downstream of mAChR signal transduction such as PLC and protein kinase C (PKC), despite no changes in M₃mAChR protein expression. This study also presented high parasympathetic activity, which was associated with metabolic malfunction, including beta cell impairment [50]. As was previously reported by our group, MSG-obese mice also have high vagus nerve activity [16], which could be related to the beta cell dysfunction that is associated with disrupted normal mAChR subtype composition/function.

Our data are therefore consistent with the conclusion that mAChRs, especially the M₁ and M₃ subtypes, are involved in the metabolic disturbances that are observed in MSG-obese rats. However, other proteins are likely involved in the downstream insulinotropic cholinergic response and may be additional targets of the impaired glucose- and insulin-coupled homeostasis processes. Nevertheless, further studies are needed to explain the other mechanisms that may be involved in altered mAChR modulation during ANS imbalance. These studies might yield data that are important for pharmacological treatment of type 2 diabetes and associated disorders.
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Conflict of Interest

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