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# Impaired muscarinic type 3 (M3) receptor/PKC and PKA pathways in islets from MSG-obese rats

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**Abstract** Monosodium glutamate-obese rats are glucose intolerant and insulin resistant. Their pancreatic islets secrete more insulin at increasing glucose concentrations, despite the possible imbalance in the autonomic nervous system of these rats. Here, we investigate the involvement of the cholinergic/protein kinase (PK)-C and PKA pathways in MSG β-cell function. Male newborn Wistar rats received a subcutaneous injection of MSG (4 g/kg body weight (BW)) or hyperosmotic saline solution during the first 5 days of life. At 90 days of life, plasma parameters, islet static insulin secretion and protein expression were analyzed. Monosodium glutamate rats presented lower body weight and decreased nasoanal length, but had higher body fat depots, glucose intolerance, hyperinsulinemia and hypertrigliceridemia. Their pancreatic islets secreted more insulin in the presence of increasing glucose concentrations with no modifications in the islet-protein content of the glucose-sensing proteins: the glucose transporter (GLUT)-2 and glycokinase. However, MSG islets presented a lower secretory capacity at 40 mM $K^+$ ($P < 0.05$). The MSG group also released less insulin in response to 100 μM carbachol, 10 μM forskolin and 1 mM 3-isobutyl-1-methyl-xantine ($P < 0.05$, $P < 0.0001$ and $P < 0.01$). These effects may be associated with a the decrease of 46 % in the acetylcholine muscarinic type 3 (M3) receptor, and a reduction of 64 % in PKCα and 36 % in PKAα protein expressions in MSG islets. Our data suggest that MSG islets, whilst showing a compensatory increase in glucose-induced insulin release, demonstrate decreased islet M3/PKC and adenylate cyclase/PKA activation, possibly predisposing these prediabetic rodents to the early development of β-cell dysfunction.

**Keywords** Insulin secretion · Monosodium glutamate · Obesity · M3 · PKC · PKA

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## Introduction

Insulin secretion from pancreatic β-cells is regulated by several factors including fuels, hormones, and neurotransmitters. These agents modify the intracellular concentrations of several β-cell regulators such as $Ca^{2+}$, phospholipids, and cyclic nucleotides that influence the amplitude and profile of the insulin secretory response [1].

During insulin resistance the endocrine pancreas develops a compensatory increase in β-cell function and, over extended time periods, an increase in β-cell numbers that enhances insulin secretion, thereby preventing hyperglycemia [2, 3]. Consequently, an adequate regeneration of β-cell intracellular messengers, following glucose or

neuro-hormonal stimuli, contributes to islet secretory function and survival [1, 4].

Early postnatal monosodium L-glutamate (MSG) administration in rodents results in neuronal necrosis within the preoptic and arcuate nuclei of the hypothalamus, leading to the development of obesity in adulthood [5, 6]. Monosodium glutamate rodents present normophagia [7], massive amounts of body fat, glucose intolerance, hyperinsulinemia and insulin resistance [8]. Pancreatic islets from obese-MSG rodents secrete high levels of insulin in response to glucose [9–11]; however, a possible disruption in autonomic nervous system (ANS) action in obesity is reported [8, 9, 12, 13]. Monosodium glutamate mice demonstrate increased vagus nerve firing and decreased sympathetic activity [14]. In addition, adrenal catecholamine synthesis, storage and secretion are reduced in MSG mice [7]. Vagotomy or physical exercise, which enhance sympathetic activity, decrease body fat depots, hyperinsulinemia, improve glucose tolerance and β-cell responsiveness to fuels in different experimental models [8, 10, 14, 15]. The molecular pathways involved in MSG β-cell secretory capacity are poorly investigated. Here, we evaluated insulin secretion in response to depolarization and, for the first time, following M3 receptor/PKC and PKA activation in islets isolated from 90-day-old MSG rats.

## Materials and methods

### Materials

$^{125}$I human insulin was purchased from Amersham International (Little Chalfont, Bucks, UK). Routine reagents were purchased from Sigma Chemical (St Louis, MO, USA).

### Animals and MSG treatment

All experiments were approved by the University's Committee on Ethics in Animal Experimentation (CEEAAP/UNIOESTE). Male newborn Wistar rats received a subcutaneous injection of MSG (4 g/kg BW/day, MSG group), or hyperosmotic saline solution (1.25 g/kg BW/day, CTL group), during the first 5 days of life [9]. Pups were weaned on the 21st day of life. All animals had free access to standard rodent chow (Nuvital, Colombo, Brazil) and water; they were housed in standard cages and maintained on a 12 h light/dark cycle (lights on 06:00–18:00 h) and controlled temperature (22 ± 1 °C).

### Obesity and biochemical parameters

At 90 days of age, both rat groups were weighed and nasoanal lengths were measured to obtain the Lee Index [from the ratio of body weight (g)$^{1/3}$/Nasoanal length (cm) × 1,000] for use as a predictor of obesity in rodents [16]. Retroperitoneal and periepididymal fat pads were removed and weighed. Fasted rats were decapitated, their blood collected and plasma was stored at −20 °C for determination of insulin by radioimmunoassay (RIA), as previously reported [17]. Plasma glucose was measured from the tail tip using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland). Total cholesterol (CHOL) and triglycerides (TG) were measured using standard commercial kits, according to the manufacturer's instructions (Boehringer Mannhein®, Germany; Merck®, Germany).

### Intravenous glucose tolerance test (ivGTT) and insulin sensitivity

At 90 days of life, MSG and CTL rats were anesthetized with thiopental via ip (1 mg/kg BW) to implant a silastic cannula into the right jugular vein [18]. After an overnight fast, glucose (1 g/kg BW) was administered into the cannula. Blood samples were collected in heparinized syringes at 0 (before glucose administration), 5, 15 and 30 min for plasma glucose measurement. After each bleeding, the equivalent volume of 0.9 % saline solution was injected. Plasma was stored at −20 °C for posterior plasma glucose concentration measurement by the glucose oxidase method [19]. Tissue insulin sensitivity was also evaluated by the previously validated [20] homeostasis model assessment (HOMA), using the HOMA index of insulin resistance (HOMA-IR) = fasting insulin (μU/mL) × fasting glucose (mM)/22.5, described by Matthews et al. [21].

### Islet isolation and static insulin secretion

Islets were isolated by collagenase digestion of the pancreas. For static incubations, four islets from each group were first incubated for 30 min at 37 °C with 1 mL Krebs–bicarbonate (KBR) buffer with the following composition: 115 mM NaCl, 5 mM KCl, 2.56 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 15 mM HEPES, supplemented with 5.6 mM glucose, 3 g of BSA/L, and equilibrated with a mixture of 95 % $O_2$/5 % $CO_2$ to give pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for 1 h with 2.8, 5.6, 8.3 and 16.7 mM glucose or were incubated in the presence of 2.8 mM glucose without or with 40 mM $K^+$, 10 mM L-arginine (Arg) or 10 mM L-leucine (Leu). In another series of experiments, insulin secretion was evaluated in response to 8.3 mM glucose without or with 100 μM carbachol (Cch), 100 nM phorbol 12-myristate 13-acetate (PMA), 10 μM forskolin and 1 mM 3-isobutyl-1-methyl-xantine (IBMX). Aliquots of the supernatant were collected at the end of the incubation

period and kept at −20 °C for posterior insulin measurement by RIA, as previously reported [17]. For islet insulin content, groups of 4 islets were collected and transferred to tubes of 1.5 mL. Deionized water (1 mL) was added to the samples, followed by disruption of the pancreatic cells using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY, USA) and the islet insulin content was also measured by RIA.

### Western blot

For protein expression experiments, groups of islets isolated from MSG and CTL groups were solubilized in homogenization buffer (100 mM Tris pH 7.5, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 1 % Triton-X 100) at 4 °C using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY, USA). The extracts were then centrifuged at 12,600×*g* (Eppendorf 5417R, New Jersey, USA) at 4 °C for 5 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method (Bradford, 1974), using BSA as a standard curve and a commercial Bradford reagent (Bio-Agency Lab., São Paulo, SP, BRA). For SDS gel electrophoresis and Western blot analysis, the samples were homogenized with a loading buffer containing dithiothreitol. After heating at 95 °C for 5 min, the proteins were separated by electrophoresis (50 μg protein/lane in 10 % gels) and then transferred to nitrocellulose membranes. The membranes were subsequently blotted with specific primary antibodies against GLUT-2 (1:1,000; cat no 400061), GCK [1:1,000; cat no sc (H-88) 7908], M3 (1:200; cat no sc-9108), PKCα (1:1,000; cat no sc-8393) and PKAα (1:1,000; cat no sc-903). With the exception of GLUT-2 (Calbiochem, San Diego, CA, USA), all primary antibodies were purchased from Santa Cruz Biotechnologies, CA, USA. Visualization of specific protein bands was carried out by incubating the membranes with appropriate secondary antibodies (1:10,000; Zymed Laboratories, Inc., CA, USA), followed by exposure to X-Ray films. The band intensities were quantified by optical densitometry using the free software, Image J (http://rsbweb.nih.gov/ij). After assaying the target proteins, Western blotting was repeated using a rabbit polyclonal antibody to β-actin protein (1:10,000; cat no ab8227; Abcam, Cambridge, MA, USA) as an internal control.

### Statistical analysis

Results are presented as mean ± SEM for the number of determinations (*n*) indicated. Statistical analyses were performed using unpaired Student's *t* test; the level of significance was set at $P \leq 0.05$. Analyses were performed using Graph-Pad Prism version 5.00 for Windows (GraphPad® Software, San Diego, CA, USA).

**Table 1** General characteristics and biochemical parameters in fasted MSG and CTL (90-day-old) male Wistar rats

| | CTL | MSG |
|---|---|---|
| BW (g) | 362 ± 21 | 312 ± 10* |
| Nasoanal Length (cm) | 22.0 ± 0.2 | 19.5 ± 0.2* |
| Lee Index | 324 ± 8 | 348 ± 3* |
| Retroperitoneal fat pad (g/100 g BW) | 1.05 ± 0.34 | 2.93 ± 0.18* |
| Perigonadal fat pad (g/100 g BW) | 1.33 ± 0.42 | 3.66 ± 0.16* |
| Glucose (mg/dL) | 81 ± 8 | 88 ± 9 |
| Insulin (pg/mL) | 302 ± 40 | 853 ± 52* |
| HOMA-IR | 1.31 ± 0.13 | 5.55 ± 0.66* |
| TG (mg/dL) | 68.8 ± 6.8 | 151 ± 9.7* |
| CHOL (mg/dL) | 60.36 ± 2.80 | 60.38 ± 2.83 |

Data are mean ± SEM ($n = 11$–13)

* $P < 0.05$ vs CTL

## Results

### Animal characteristics

At 90 days of age, MSG rats presented a lower body weight and nasoanal length, compared to CTL rats ($P = 0.03$ and $P < 0.0001$, respectively; Table 1). Monosodium glutamate rats also developed obesity, since the Lee index and body fat depots are higher in MSG than CTL rats ($P < 0.01$ and $P < 0.001$, respectively; Table 1). Monosodium glutamate rats were normoglycemic due to hyperinsulinemia and presented increased plasma TG concentrations compared with those of CTL rats ($P < 0.0001$; Table 1). CHOL plasma levels were similar between groups.

Both rat groups were also submitted to ivGTT. After a glucose load, plasma glucose concentrations reached maximal levels at 5 min and returned to basal levels at 30 min (Fig. 1a). Monosodium glutamate rats showed increased glycemia at 5, 15 and 30 min of the test compared with CTL rats ($P < 0.001$, $P < 0.004$ and $P < 0.005$). Glucose intolerance in the MSG group was also confirmed by the higher area under the curve (AUC) of glycemia during ivGTT, compared with CTL ($P < 0.002$; Fig. 1b). In addition, MSG rats were insulin resistant, since HOMA-IR was higher in this group than CTL ($P = 0.0008$; Table 1).

### Glucose-induced insulin secretion and islet insulin content

Figure 2a shows the glucose-induced insulin secretion in isolated islets from MSG and CTL rats. Despite their similar

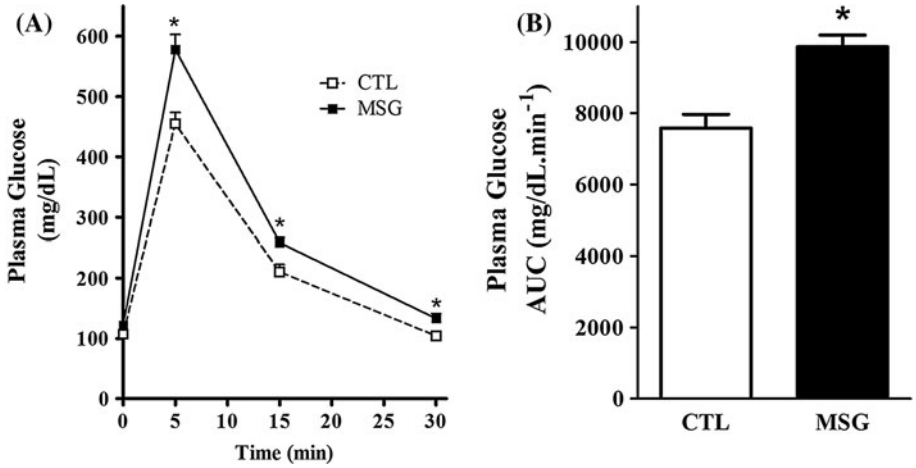


**Fig. 1** **a** Changes in plasma glucose concentrations during ivGTT in CTL and MSG rats. **b** Mean ± SEM of AUC of glycemia during the ivGTT (CTL = 11 and MSG 11). *$P < 0.05$ vs CTL

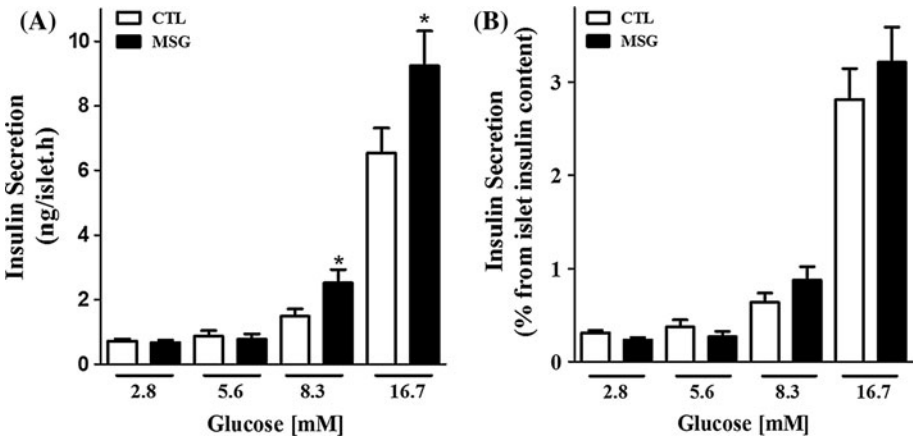


**Fig. 2** **a** Glucose-induced insulin secretion in islets isolated from MSG and CTL rats. Groups of 4 islets were incubated for 1 h with different glucose concentrations, as indicated. **b** Insulin release normalized by islet insulin content. Each bar represents mean ± SEM (CTL = 17 and MSG = 19 groups of islets). *Represents significant difference between MSG and CTL islets at the same glucose concentration ($P < 0.05$)

insulin releases at basal conditions, MSG islets secreted more insulin in response to 8.3 and 16.7 mM glucose, compared with CTL islets ($P < 0.04$ and $P < 0.03$). However, the MSG islets secreted less insulin in response to a depolarizing stimulus of 40 mM $K^+$ ($P < 0.05$; Fig. 3a). Interestingly, in the presence of 10 mM Arg, MSG islets secreted more insulin than CTL islets ($P < 0.0001$; Fig. 3a). The release of insulin in response to 10 mM Leu was similar in the MSG and CTL islets (Fig. 3b).

Total insulin content in MSG islets was 19 % higher than in CTL islets (287 ± 19 and 232 ± 11 ng/islet, respectively; $P < 0.02$). When insulin secretion in response to increasing glucose concentrations was expressed as the percentage of hormone release from islet content, MSG and CTL islets showed a similar insulin secretory capacity (Fig. 2b).

### Cch, Forskolin and IBMX-potentiated insulin secretion

When islets were incubated in the presence of 100 μM Cch, insulin secretion was lower in MSG than in CTL islets (Fig. 4a; $P < 0.05$). At 100 nM PMA, insulin release was similar between MSG and CTL islets (Fig. 4a). However, PMA elicited an increase of 7 % in insulin secretion in MSG islets, while in CTL islets, the PKC activator, enhanced 54 % the hormone release. In addition, the increases in insulin release elicited by 10 μM Forskolin and 1 mM IBMX (which increase cAMP levels by AC stimulation or by phosphodiesterase inhibition, respectively) were reduced in MSG islets, compared with CTL islets ($P < 0.0001$ and $P < 0.01$ respectively; Fig. 4b).

### GLUT-2, GCK, M3, PKCα and PKAα protein expressions

Western blot analyses showed that the β-cell glucose sensor proteins, GLUT-2 and GCK, did not differ between the groups of islets (Fig. 5a, b). However, the M3 receptor, PKCα and PKAα protein contents were 46, 64 and 37 % lower in MSG islets, compared with CTL islets ($P < 0.05$, $P < 0.0001$ and $P < 0.03$, respectively; Fig. 5c–e).

## Discussion

Islets isolated from MSG-obese rats secreted more insulin in response to 8.3 and 16.7 mM glucose (Fig. 2), supporting

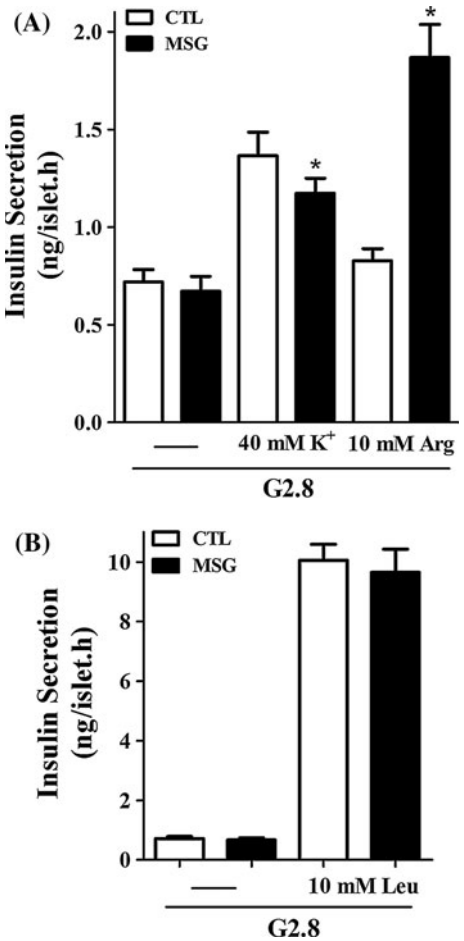


**Fig. 3** Insulin secretion in the presence of 2.8 mM glucose (G2.8) without or with **a** 40 mM $K^+$, 10 mM Arg or **b** 10 mM Leu from islets isolated from MSG and CTL rats. Each bar represents the mean ± SEM (CTL = 18 and MSG = 20 groups of islets). *Represents significant difference between MSG vs CTL islets with the same stimulus, $P < 0.05$

previous observations [10, 11]. This evidence demonstrates a potential adequate adaptation to insulin resistance and glucose intolerance in these rats (Fig. 1; Table 1). In contrast, when insulin release was normalized by the islet insulin content, MSG and CTL islets showed similar secretions, this observation suggests that the MSG group did not present any alteration in islet glucose responsiveness; however our additional incubation experiments showed an impaired secretory response in this group. Monosodium glutamate islets release less insulin when challenged by a depolarizing stimulus (Fig. 3a). Since insulin secretion in response to 40 mM $K^+$ only comprises the $K^+$-dependent pathway of biphasic insulin release [22], our result suggests that the mechanisms involved in the first phase of secretion are impaired in MSG islets. Unfortunately, this observation contradicts that reported by Grassioli et al. [23], who found that only the second phase of the insulin secretion was damaged in MSG rats. These discrepancies may be due to the fact that the study by Grassioli et al., observed insulin release only in response to a depolarizing stimulus plus diazoxide. Interestingly, Arg induced a higher insulin secretion in MSG islets, compared with CTL islets (Fig. 3a), suggesting that the glutamate pool, generated by Arg metabolism, makes a large contribution to insulin secretion, since this metabolite facilitates insulin granule exocytosis [24].

Several reports have shown that increased vagal cholinergic activity and reduced sympathetic tone are present in different experimental models of prediabetes and diabetes [9, 12–14]. It is known that β-cell function is modulated by cell membrane receptors that are activated by neurotransmitters and hormones [1, 4]. The pancreatic islets are richly innervated by parasympathetic terminals of the vagus [4]. Acetylcholine (Ach) released by these terminals acts mainly upon M3 receptors that stimulate Gq proteins, which in turn activate different isoforms of PLC. This enzyme acts upon membrane phosphatidylinositol 4,5-bisphosphate (PIP2), generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), subsequently activating PKC and increasing $Ca^{2+}$ mobilization from intracellular stores, respectively [4]. In addition, stimulation of the β-cell M3 receptor partially depolarizes the plasma membrane via activation of $Na^+$ channels that sustain the increase in intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]i$) following glucose stimulus [25].

Monosodium glutamate islets possess decreased M3 receptor protein content (Fig. 5c). This evidence is in accordance with the lower insulin secretion induced by 100 μM Cch (Fig. 4a). This impairment in β-cell cholinergic action may be due to an imbalance of ANS in obese-MSG rats that may lead to the down-regulation of M3 receptor content in the β-cells, since high acetylcholinesterase activity was present in the pancreas and in the liver of MSG mice [9]. The vagus-firing rates were higher in the MSG mice, accompanied by a decrease in sympathetic nerve activity and lower adrenal catecholamine stores [14]. In addition, subdiaphragmatic vagotomy increased Cch-induced insulin secretion in islets from obese-MSG rats [10] and improved fasting insulinemia, glucose tolerance and insulin sensitivity in obese rats [8]. Furthermore, Grassiolli et al. [26] demonstrated that M2 receptors (non-common muscarinic receptor expressed in β-cells) are active in MSG islets and that the inhibitory action of the M2 receptor may be a mechanism by which Ach decreases its insulinotropic effect, since specific blockade of M2 receptor by methoctramine increases the effects of Ach upon glucose-induced secretion in MSG islets. As such, alterations in the amounts and/or composition of the expression of muscarinic receptor subtypes could participate in the effects of Ach on β-cells under different metabolic conditions, such as obesity.

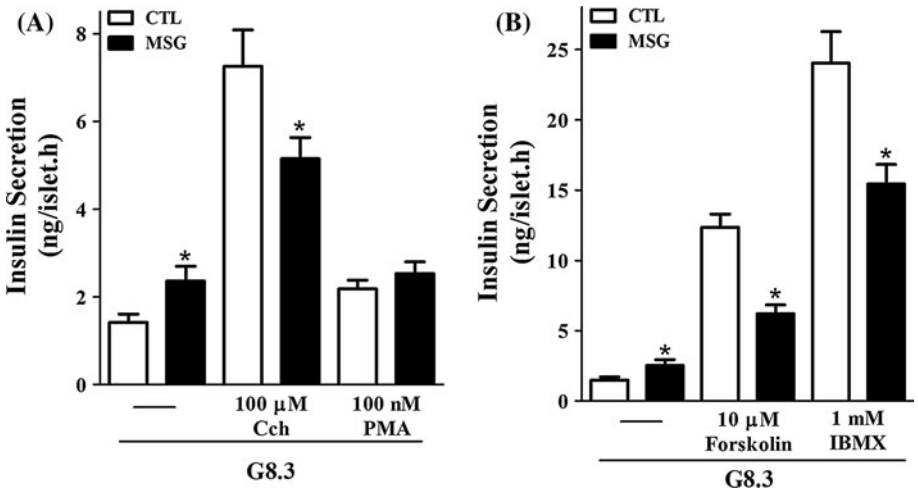


**Fig. 4** Insulin secretion induced by 100 µM Cch and 100 nM PMA **a** or in response to 10 µM forskolin or 1 mM IBMX **b** in islets from MSG and CTL rats. Groups of 4 islets were incubated for 1 h at 8.3 mM glucose (G8.3), with or without Cch, forskolin or IBMX. Each bar represents mean ± SEM (CTL = 12 and MSG = 14).*$P < 0.05$ related to respective CTL

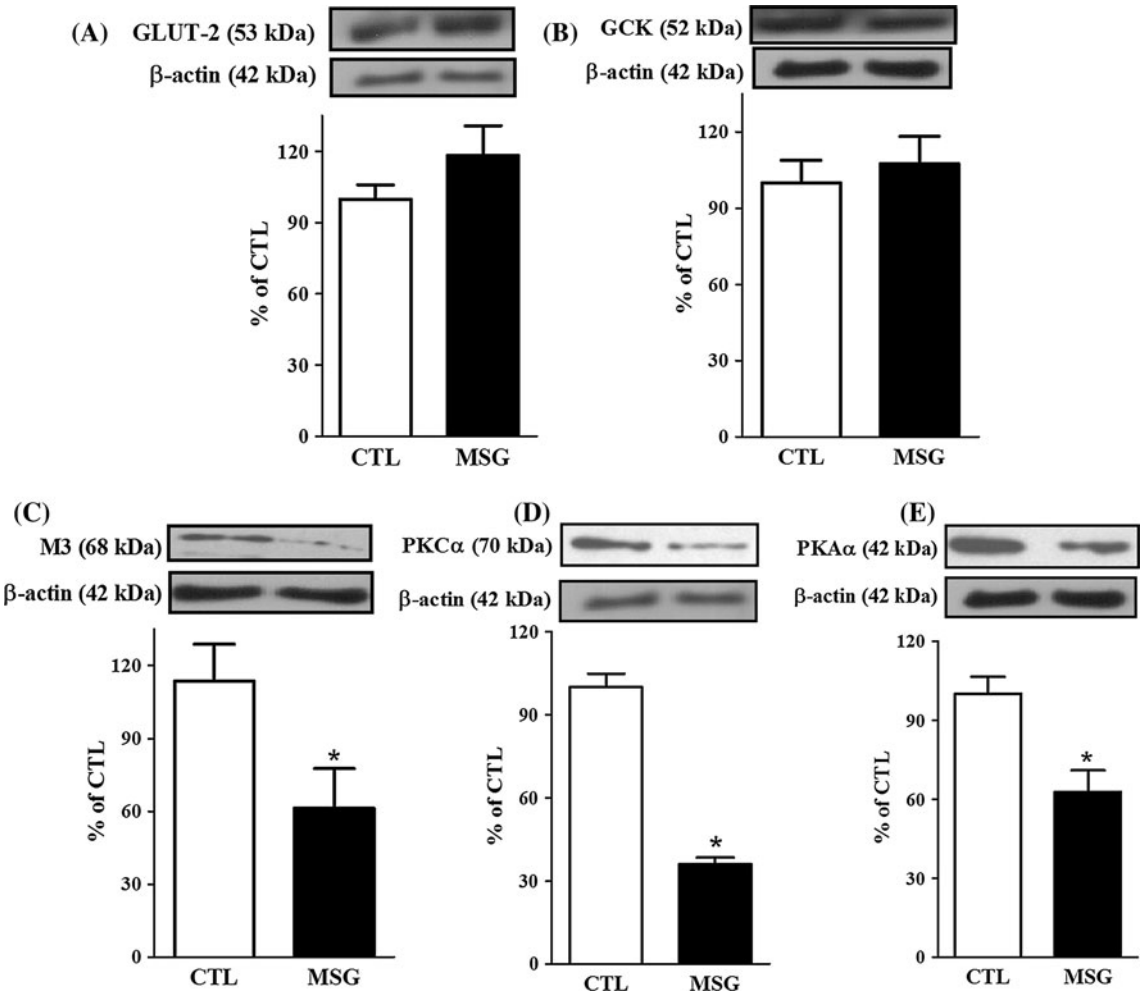


**Fig. 5** GLUT-2, GCK, M3, PKCα and PKAα protein expressions in islets from MSG and CTL rats. Protein extracts were processed for Western blot detection of GLUT-2 (**a**), GCK (**b**), M3 (**c**), PKCα (**d**), PKAα (**e**) and β-actin (internal control). Bars represent the mean ± SEM of the values, determined by optical densitometry ($n = 3$–8 rats). *$P < 0.05$ related to CTL

The physiological role of M3 receptors in glucose homeostasis has been widely studied by Gautam's group, who found that, in β-cell M3-receptor knockout mice, an impaired glucose tolerance and decreased insulin secretion are noted [27]. In contrast, transgenic mice that overexpress M3 receptors only in β-cells showed enhanced

glucose tolerance and insulin release and were resistant to diet-induced damage to glucose control [27]. Thus, evidence indicates that the decreased M3 receptor and PKCα protein content in MSG islets (Fig. 5c, d) may be involved in the impaired glucose control in these obese rats.

It is also known that β-cells express different types of AC, which may account for the increase in cAMP levels and for the increase in $[Ca^{2+}]i$ [28, 29]. cAMP activates PKA, which phosphorylates several proteins, resulting in enhanced $[Ca^{2+}]i$, insulin granule exocytosis and maintenance of β-cell mass [30, 31]. Decreased PKAα protein expression (Fig. 5e) may contribute to decrease β-cell function in obese-MSG rats and may contribute to the loss of islet tissue and development of diabetes. Moreover, both the reduction in M3/PKCα content and the lower PKAα expression may result in a reduced parasympathetic action in MSG islets, since it was shown that both PKC and PKA are involved in the effects of Ach upon β-cells. Ach restores the insulin secretory glucose competence of Goto-Kakizaki (GK) rats via activation of PKA [32]; in GK islets, Ach induces first phase insulin release at low glucose, but has no effect in islets from Wistar rats. This effect was also present when PKC was inhibited and thapsigargin was used to disrupt the Ach-induced $[Ca^{2+}]i$ increase. However, inhibition of PKA in GK islets leads to a significantly lower insulinotropic response to Ach [32]. In this way, since incretin levels are significantly decreased in type 2 diabetes [33] and PKA islet protein content was reduced in prediabetic states [34, 35]. These factors contribute to decline β-cell function and our data showing lower insulin secretion and PKA in MSG obese-rats indicate early steps in the development of islet dysfunction.

## Conclusions

For the first time, we demonstrated that MSG islets have decreased M3 receptor, PKCα and PKAα protein expression. Consequently, some impairment of β-cell function appears by 90 days of age in these obese rats, despite an enhanced secretory capacity following glucose stimulus. In addition, since these receptors and kinases (besides their importance in insulin secretion amplification in response to glucose and adequate granule exocytosis) are also crucial for islet mass preservation [36], our data suggest that MSG islets may develop β-cell dysfunction and failure during the maintenance of the endocrine pancreas tissue, leading to the development of diabetes.

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**Conflict of interest** The authors have no conflict of interest.

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