ORIGINAL ARTICLE

Swim training of monosodium L-glutamate-obese mice improves the impaired insulin receptor tyrosine phosphorylation in pancreatic islets

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Abstract The goal of the present study was to investigate changes on glucose homoeostasis and of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) signalling in pancreatic islets from MSG-obese mice submitted to or not submitted to swim training. Swim training of 90-day-old MSG mice was used to evaluate whether signalling pathways of the IR and IRS-1 in islets are involved with the insulin resistance and glucose intolerance observed in this obese animal model. The results showed that IR tyrosine phosphorylation (pIR) was reduced by 42 % in MSG-obese mice (MSG, 6.7 ± 0.2 arbitrary units (a.u.); control, 11.5 ± 0.4 a.u.); on the other hand, exercise training increased pIR by 76 % in MSG mice without affecting control mice (MSG, 11.8 ± 0.3; control, 12.8 ± 0.2 a.u.). Although the treatment with MSG increased IRS-1 tyrosine phosphorylation (pIRS-1) by 96 % (MSG, 17.02 ± 0.6; control, 8.7 ± 0.2 a.u.), exercise training also increased it in both groups (control, 13.6 ± 0.1; MSG, 22.2 ± 1.1 a.u.).

Current research shows that the practice of swim training increases the tyrosine phosphorylation of IRS-1 which can modulate the effect caused by obesity in insulin receptors.

Introduction

Obesity is becoming one of the most important increasing physiopathological conditions worldwide. Insufficient physical activity and excessive caloric intake are the causes for increasing body weight [1, 2]. The obesity produced by neonatal treatment with monosodium L-glutamate (MSG) is a major tool to study the effects of obesity on different metabolic parameters. This model is characterised by hyperinsulinaemia and higher insulin secretion [3] or early hyperglycaemia in MSG-treated mice [4, 5].

MSG-obese rodents present a severe tissue insulin resistance, which is observed as a dysfunction of the insulin receptor (IR) as well as in internal signal transduction pathways [6, 7]. When activated by insulin, the IR stimulates several intracellular substrates, such as insulin receptor substrate-1 (IRS-1), which play a key role in transmitting signals to intracellular pathways [8, 9]; multiple tyrosine residues of IRS-1 themselves are then phosphorylated by these receptors, which plays an important role in their biological function. Besides the insulin effect on peripheral tissues, mostly adipose and muscle, islet cells are directly influenced by insulin.

In recent years, autocrine effects of insulin on beta cells have remained controversial. Recently, however, a variety of new methods have been utilised to demonstrate the strong clinical importance of the autocrine actions of

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insulin [10, 11]. It is accepted that insulin also possesses auto- and paracrine actions in pancreatic islets cells [12]. While most of the metabolic actions of insulin are exerted in the peripheral tissues, few studies have attempted to clarify the early events in the insulin-signalling pathways. In 1995, Velloso et al. [12] showed glucose and insulin-induced phosphorylation of IRS-1 and IRS-2 in rat pancreatic islets. However, the mechanisms involved in insulin signalling in the islets are currently unknown.

Studies have shown that defects in glucose homoeostasis in obese rodents and humans are improved by exercise training [13–16]. Glucose transport stimulated by insulin in the peripheral tissues during exercise training is not increased by the expression of components of the insulin-signalling cascade but is rather due to insulin activating other insulin transduction pathways [17]. It was also reported that exercise training improves beta cell function and mass by enhancing IRS-2 expression in the islets of diabetic rats [18]. However, it is not known exactly which mechanisms of insulin signalling are involved in these effects.

In this study, we used a well-established hyperglycaemic and hyperinsulinaemic obese and insulin-resistant experimental model, MSG mice, to determine whether the signalling pathways of IR and IRS-1 in the pancreatic beta cell are involved. Swim training of MSG-induced obese mice was used to observe changes in glycaemic homoeostasis as well as insulin signalling in pancreatic islets.

Materials and methods

Animals and obesity treatment

All animal protocols were approved by the Animal Ethics Committee of the State University of Minas Gerais. Neonate male *Mus musculus* Swiss mice were subcutaneously injected during the first 5 days of life with MSG at a dose of 4 mg/g body mass (BM). Control animals received a saline solution. Both animal groups (15 mice from 3 litters of each group) were weaned at the 21st day of life. All animals were housed under controlled conditions in a 12 h light–dark cycle of (07:00–19:00 h) and temperature (21 ± 2 °C). Water and standard rodent chow (Nuvital–Curitiba–Brazil) were offered ad libitum.

At weaning, only males were selected for the experimental procedures. Control and MSG-obese mice were randomly chosen for exercise training.

Swimming training

Control (Cont EXE) and MSG-obese (MSG EXE) mice were trained by free swimming in a glass tank (30 × 35 × 30 cm) filled with water at 32 ± 3 °C as reported by Scomparin et al. [19]. Mice were exercised for 15 min/day, three times per week over a period of 69 days. Four mice from each group were placed simultaneously into the pool at (2:00 p.m.). Additional animal groups, sedentary control and MSG-obese, did not swim (Cont SED and MSG SED, respectively).

The swimming programme should be considered a light exercise when compared to a previously reported moderate training programme in which mice freely swam for 60 min, 5 days a week, for 18 weeks [20]. After each exercise, mice were dried with paper towels and returned to their respective cages until the next swimming session as described by Andreazzi et al. [5].

Intraperitoneal glucose tolerance test (ipGTT)

ipGTT was performed by injecting glucose (2 g/kg BM) i.p. in overnight-fasted mice. Blood glucose levels were determined prior (0) to injection and 30, 60, 90, and 120 min after injection. Blood samples were obtained from the tail vein and plasmas were used to measure glucose concentration by the glucose oxidase method.

The total area-under-the-curve of ipGTT was calculated.

Assessment of obesity

All animals were weighed weekly and were killed at 90 days of age by cervical dislocation after being anaesthetised with thiopental (45 mg/kg BM). Obesity was assessed by BM and nasoanal length to calculate the rodent body mass index or Lee index. The Lee index was calculated from the ratio [$BM^{1/3}$ (g)/nasoanal length (cm)] [21]. The epididymal fat was removed and weighed only for being used as a simple and reliable parameter of body fat in normal and obese rodents [22].

Pancreatic islet isolation

Isolation of pancreatic islets from mice was performed with a method adapted from one previously described [23]. Animals were deprived of food the evening before 12 h they were used for islet preparation. After anaesthesia as previously described, the abdominal wall of intact mice of all groups was cut open. A 10 ml Hank's buffered saline solution (HBSS) containing 0.1 % collagenase type XI (Sigma Chemical Co., St. Louis, MO) was injected into the common bile duct. The pancreas, swollen with the collagenase solution, was quickly excised and incubated in a plastic culture bottle for 16 min at 37 °C. After that, 4 continuous washings were made in HBSS containing 0.12 % bovine serum albumin fraction V (BSA). Islets were collected with the aid of a microscope.

Insulin secretion stimulation

Isolated islets adapted to a baseline glucose concentration (5.6 mmol/l) were pre-incubated in 1 ml of normal Krebs–Ringer solution [(mmol/l): NaCl, 115; NaHCO$_3$, 24; KCl, 1.6; MgCl$_2$·6H$_2$O, 1; CaCl$_2$·2H$_2$O, 1; BSA, 15] containing 5.6 mmol/l glucose, for 60 min. This solution was gassed with (O$_2$, 95 % + CO$_2$, 5 % mixed) to maintain pH 7.4. After adaptation to low glucose concentration, islets were incubated for a further 60 min in glucose 5.6 and 16.7 with Krebs–Ringer solution. The origin of islets was from at least ten different mice to each experimental group. Aliquots from incubations were used to measure insulin concentration by RIA.

Western blotting

After isolation, groups of islets were pelleted by centrifugation at 12,000 rpm at 4 °C for 20 min and resuspended in 50–100 μl of homogenisation buffer containing protease inhibitors [24, 25]. The islets were sonicated and the total protein content was determined by the Bradford method (Bio-Rad®). Samples containing 100 mM DTT and 90 μg of protein were treated with Laemmli buffer (1 M sodium phosphate/l, pH 7.0, 0.1 % bromophenol blue, 50 % glycerol, 10 % SDS and water) and then electrophoresed on an 8 % SDS-polyacrylamide gel at 200–400 mA for 90 min. (Mini-Protein Bio-Rad®).

Electrotransfer of proteins to nitrocellulose membranes was performed in a buffer containing 25 mM Tris–Base, 192 mM glycine, 20 % methanol and SDS 0.02 % in sufficient quantity water to 1,000 ml for 2 h. After checking the transfer efficiency by Ponceau S staining, the membranes were blocked with 5 % skimmed milk in Tween-Tris buffered saline (TTBS; 10 ml of 1 M Tris–base; 30 ml of 5 M NaCl, 500 μl of Tween 20 and 1,000 ml water). The membrane was incubated with primary antibodies epitope corresponding to phosphorylated Tyr IR (pIR) and IRS-1 (pIRS-1) (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; diluted 1:1,000 in TTBS containing 1 % dry skimmed milk) overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h (Amersham-Pharmacia Biotech, Buckingham Shire, UK; diluted 1:10,000 in TTBS containing 1 % dry skimmed milk). The results were visualised by fluorescent immunodetection in combination with the Molecular Dynamics™ Storm™ imaging system. Band intensities were quantified by optical densitometry (Hoefer Scientific Instruments, GS300). The β-actin protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; diluted 1:1,000 in TTBS containing 1 % dry skimmed milk) was used for normalisation.

Statistical analysis

Results are reported as mean ± SEM and were analysed with Student's t tests or one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc tests. p values less than 0.05 were considered statistically significant. Tests were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software®).

Results

The evolution of BM from 21 to 90 days of age, the Lee index and epididymal fat pads show that the neonatal MSG treatment was effective at inducing obesity in mice, as shown in Fig. 1A–C, respectively. Area-under-the-curve (AUC) analysis shows that the MSG SED group was 28 % smaller than the Cont SED group, whereas the MSG EXE group was 27 % smaller when compared to the MSG SED group (inset of Fig. 1A, $p < 0.001$). The MSG-treated SED group shows increase in epididymal fat pad, twofold more than CON SED group ($p < 0.0001$).

Figure 2 shows the effect of swim training on fasting plasma glucose (Fig. 2A) and insulin (Fig. 2B) concentrations. MSG treatment leads to increases in basal glucose (140.0 ± 1.8 mg/dl, MSG SED) and insulin (2.3 ± 0.1 ng/ml, MSG SED) of 9 and 139 %, respectively, when compared to the Cont SED group, basal glucose (127.9 ± 1.2 mg/dl) and insulin (0.9 ± 0.04 ng/ml). While exercise did not change fasting glucose levels of MSG-untreated mice, it decreased fasting glycaemia by 22 % (109.2 ± 0.9 mg/dl, MSG EXE) and fasting insulin by 39 % (13.9 ± 0.2 ng/ml, MSG EXE) in trained MSG mice compared to sedentary MSG mice ($p < 0.001$). Cont EXE group showed that basal glucose (125.4 ± 1.04 mg/dl) and fast insulin (0.89 ± 0.05 ng/dl) compared with the Cont SED group decrease of 1.95 and 6.08 %, respectively, were not significant.

Glucose concentrations during the ipGTT were not significantly affected by exercise training in lean animals (Cont EXE, 391 ± 14.42 mg/dl$^{-120\ \text{min}}$; Fig. 3a) when compared to sedentary animals (AUC of Cont SED, 369.1 ± 7.96 mg/dl$^{-120\ \text{min}}$). AUC during the ipGTT from the MSG SED group was 56.5 % higher than that of Cont SED group; nevertheless, exercise training reduced it by 42 % (exercised MSG mice, 335.5 ± 13.13 mg/dl$^{-120\ \text{min}}$, $p < 0.001$), when compared to untrained MSG mice, (Fig. 3b).

When the islets were stimulated with low concentrations of glucose, insulin release was lower in Cont SED group compared to other groups, as shown in Fig. 4. When glucose concentration was increased from 5.6 to 16.7 mM, islets from all groups responded by secreting more insulin.

Fig. 1 Assessment of obesity: *symbols* represent mean ± SEM of BM (**A**), Lee index (**B**) from weaning to adulthood and epididymal fat (**C**), of sedentary and training MSG-obese and control mice obtained with 15 animals for each group. *Inset* in (**A**) represents the area-under-the-curve of BM from 21 to 90 days of age. *Letters* over the *bars* represent statistical differences ($p < 0.001$) by one-way ANOVA among *a* control SED, *b* MSG SED, *c* control EXE, *d* MSG EXE
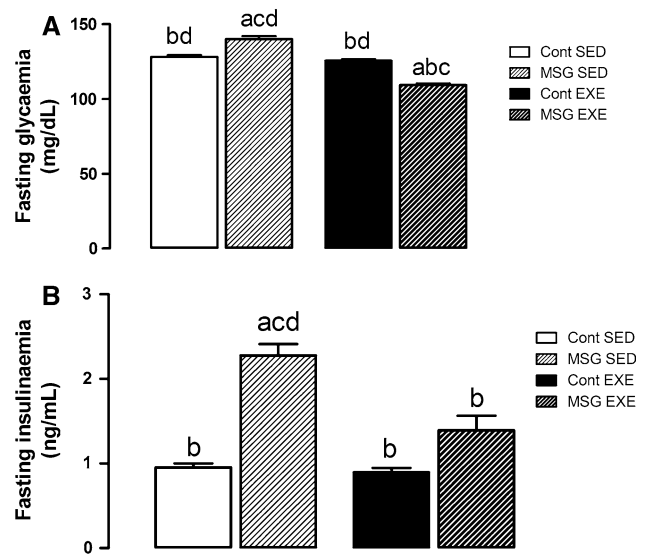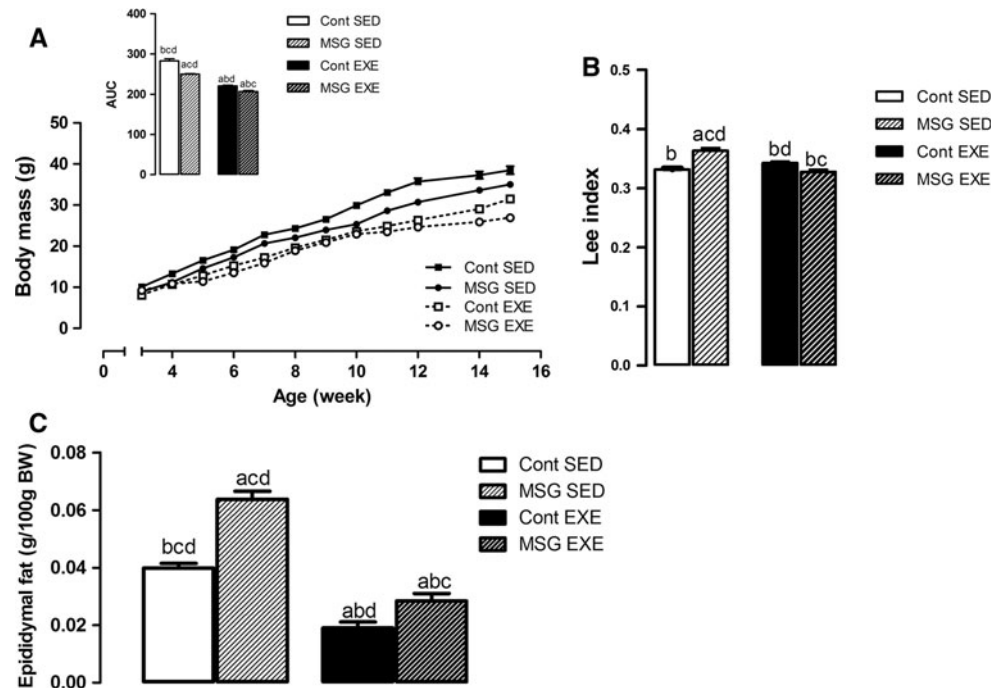

Fig. 2 Assessment of glucose homoeostasis: fasting glucose (**A**) and fasting insulin (**B**) from sedentary and swimming MSG-treated and control mice at 90 days of age for 15 animals in each group. *Letters* over the *bars* represent statistical differences ($p < 0.001$) by one-way ANOVA among *a* control SED, *b* MSG SED, *c* control EXE, *d* MSG EXE

While islets from Cont and MSG sedentary mice do not show different response to high glucose (16.7 mM), exercise training induced 128 % increase in insulin release only to MSG islets ($p < 0.001$).

While MSG treatment reduced pIR by 42 %, pIRS-1 was increased by 95 % compared to value of control mice (11.5 ± 0.4 and 8.7 ± 0.2 arbitrary units (a.u.),
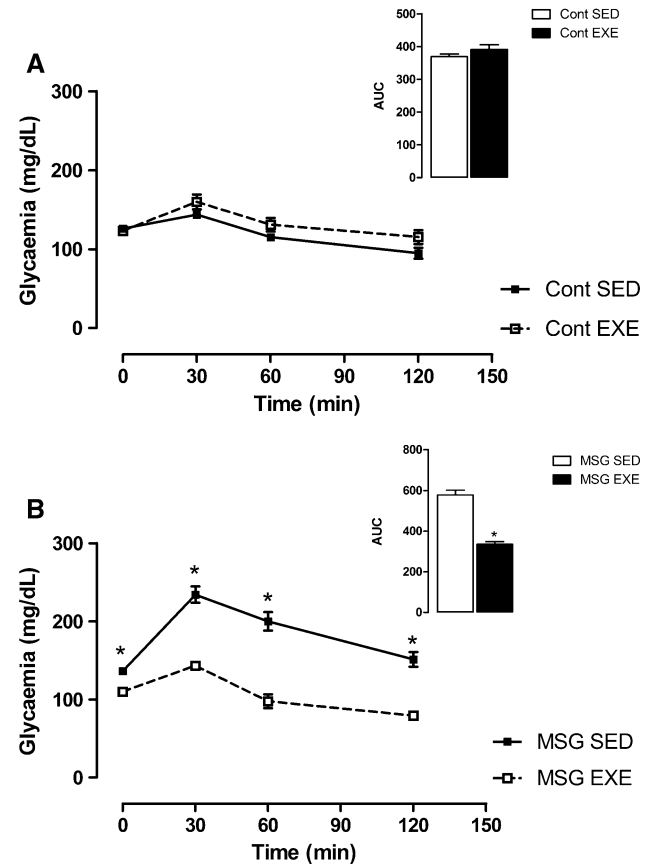
Fig. 3 Glucose tolerance test: *symbols* represent mean ± SEM of glycaemia during the ipGTT from sedentary and training control mice (**a**) and MSG-obese mice (**b**) for 15 animals in each group. *Insets* in the each figure represent the respective areas-under-the-curve of glycaemia. *$p < 0.001$ from Student's *t* test
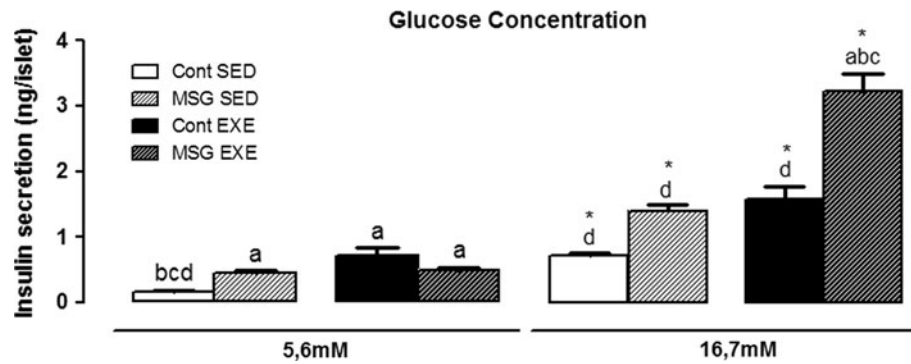
Fig. 4 Effect of MSG treatment and swimming on insulin secretion stimulated by low (5.6 mM) and high (16.7 mM) glucose concentration. Islets were obtained from a pool of 10 mice for each group. *Bars* represent mean of 20–40 batches of islets. *Letters* over the *bars* represent statistical differences ($p < 0.001$) by one-way ANOVA among *a* control SED, *b* MSG SED, *c* control EXE, *d* MSG EXE. *Asterisk* represents significant differences between insulin secretion induced by 5.6–16.7 mM for each group
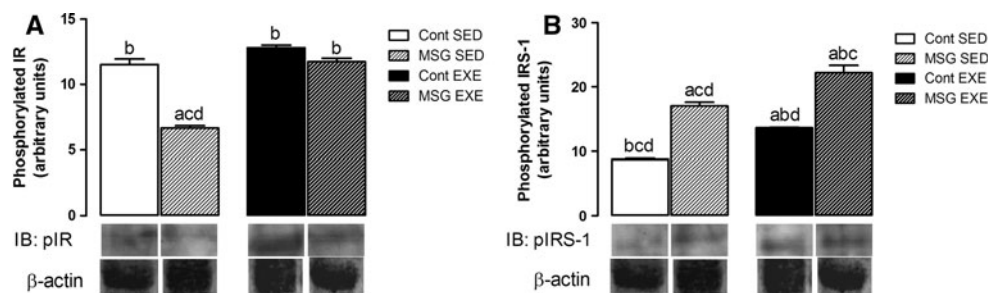

Fig. 5 Expression of phosphorylated IR and IRS-1 in islets from mice: *bars* represent mean ± SEM of the density of expression of phosphorylated IR (**A**) and IRS-1 (**B**) by IB in pancreatic islets from sedentary and training control and MSG-obese mice isolated from 15 animals for each group. *Letters* over the *bars* represent statistical differences ($p < 0.001$) by one-way ANOVA among *a* control SED, *b* MSG SED, *c* control EXE, *d* MSG EXE. *IB* immunoblotting

respectively), as shown in Fig. 5A and B. Exercise training was able to increase pIR by 76 % in MSG mice; on other hand, pIRS-1 was increased by 30 % in exercised MSG mice ($p < 0.001$). While swim training did not change pIR in control mice, pIRS-1 was enhanced by 55 % ($13.6 ± 0.1$ a.u., $p < 0.001$).

Discussion

Biometric results confirm that MSG treatment was able to induce obesity in mice in adult life. As shown previously by our laboratory [4, 5, 19] and others [26, 27], fasting glucose and insulin were higher in MSG-mediated obese mice. Exercise training normalised both insulinaemia and glycaemia as previously reported [5, 21–28]. Sedentary MSG-obese mice were highly glucose intolerant, which is attributed to impaired insulin tissue sensitivity. It has been shown that insulin resistance is an important factor in development of type 2 diabetes, as observed in the MSG experimental model [29]. Peripheral insulin resistance in obesity demands an extreme effort of β-cells to produce

and release increasing amounts of insulin despite ineffective normalisation of glycaemia.

Glycaemia of the lean exercised mice during the glucose tolerance test was not altered; however in MSG-obese mice, the fasting values were reduced by swim training. In this study, we showed that swim training is effective in the maintenance of glucose homoeostasis; as has been reported previously, insulin resistance and glucose tolerance are improved by exercise training on rodents [19, 30, 31].

In this study, the exercise effect confirms what was recently shown by our laboratory, i.e. a low-intensity swimming programme is effective at inhibiting MSG-mediated obesity in adult mice [4, 5, 19]. It is well documented that human beings and animals with metabolic syndrome undergoing physical exercise improve their blood insulin and glucose levels [32–34]. Nevertheless, with regard to the benefits of physical exercise, the mechanisms involved in the improvement of metabolism are still unclear. Although many studies have attempted to explain the mechanisms underlying exercise effects on insulin action in peripheral tissues, the effect of autocrine insulin action has not been studied. We suggest that the

reduction of insulin plasma levels in MSG-trained mice is due to both improved insulin sensitivity in peripheral tissues and effect on beta cells by increasing IR phosphorylation. Insulin binds to pancreatic islet cells [35], including beta cells [36]. IR phosphorylation was observed in beta cells [12], as well as other steps of the insulin-signalling pathway [6, 37, 38]. IR phosphorylation and downstream signal transduction in pancreatic beta cells induces activation of several genes such as mammalian target of rapamycin (mTOR), which is involved in protein biosynthesis [39]. Our data do not allow us to support the discussion about insulin biosynthesis and/or β-cell mass regulation in the MSG-obese mice, but suggest that there is an adaptation on activity of IR which down regulated the IR activity to guarantee the MSG-obese β-cell survival/preservation because its hyperinsulaemia associated to strong demand due to glucose intolerance, peripheral insulin resistance [40–43] and the high cholinergic stimulation by the parasympathetic tonus observed in this model [4, 44, 45]. In islets from MSG-obese mice, we find low pIR; however, pIRS-1 was increased. These results indicate that MSG-obese mice have lower insulin sensitivity in islets, which could be due to high activation of the intracellular signalling cascade of insulin because it was shown that protein c-Jun N-terminal kinase (JNK) was elevated in tissues from obese rodents [46]. The dysfunction in the insulin-signalling cascade could impair the insulin-induced IRS-1 phosphorylation thereby attenuating insulin sensitivity [47, 48]. It has been shown that in the obesity, insulin resistance is associated with higher phosphorylation of serine residues of IRS-1 [49, 50]. Insulin resistance in obese rodents is accompanied by hyperinsulinaemia in combination with glucose intolerance and defective insulin secretion [3, 5, 26, 51–53]. Besides a possible metabolic adaptation to preserve the β-cell in pancreatic islets suggested by our data, we do not exclude that the possibility of the lower tyrosine phosphorylated IR in islets from MSG-obese mice may be due to a higher serine phosphorylation by inflammatory processes caused by high fat accumulation [54, 55].

In type 2 diabetes, the pancreatic islets become unable to respond to increased insulin demand, secreting more and more insulin to overcome the prevailing insulin resistance [56]. However, the swim training increases pIR from MSG-obese mice compared to control mice, which maintains normal pancreatic islet sensitivity to insulin action, thereby allowing the β-cells to respond properly to the stimuli to secrete insulin. The suggested mechanism can explain, at least in part, how exercise improves and/or protects pancreatic β-cell function. Despite the exercise effect on the insulin secretion and/or biosynthesis, the glucose also exerts a positive effect on this mechanism. We speculate that there is an opposite metabolic adaptation, in which glucose has any role on overcoming the downstream

signals of IRS-1 for maintaining the storage of insulin to guarantee the high plasma insulin demand, once it is known that glucose per se in elevated concentrations has a regulatory effect on insulin secretion/biosynthesis [12, 56, 57]. Furthermore, we know the MSG-obese rats are very long hyperglycaemic which allow us to suggest that the high glucose levels exert any programmed stimulatory effect on IRS-1 activation in β-cell to maintain the insulin levels needed to peripheral glucose uptake. In this situation the exercise training has no beneficial impact on β-cell preservation, once it rises the phosphorylation of IRS-1.

Recently, we showed that glucose intolerance and insulin secretion in MSG-obese mice is improved by swim training [4, 5, 26]. It has been well established either in humans and experimental animals with type 2 diabetes that regular physical exercise enhances glucose-stimulated insulin secretion [14, 16, 58, 59] by improving peripheral insulin sensitivity [60, 61]. Beyond the peripheral effect of exercise, it has been shown that physical exercise improves β-cell function and mass by enhanced IRS-2 expression in islets of diabetic rats [18]. Although the magnitude of the increased pIRS-1 from exercised control mice was higher than MSG mice, we have shown that exercise was effective at improving pIRS-1 in isolated islets from both experimental groups, which suggests that exercise training can ameliorate pancreatic β-cell function, including insulin biosynthesis [62]. It is not surprising that insulin secretion stimulated by glucose is enhanced in islets isolated from control and exercised mice [5]. It may explain the higher pIRS-1 in the sedentary MSG-obese as a protective modulation to guarantee the insulin stores by a positive feedback mechanism [63]. In fact, it was reported that high IRS-1 activation is a protective mechanism for insulin stores from β-cells to guarantee that insulin would be synthesised and/or secreted [10]. Indeed, our results showed that exercise training used in the current work induced extra insulin release from islets of MSG mice. It must highlight that sedentary MSG mice have difficulty in secreting insulin when islets were stimulated by high glucose concentration. It has been shown that poor insulin secretion in postprandial situation is a signal to type 2 diabetes [56]. The data from insulin secretion can be associated to protein expression of IRS-1 which improves glucose-induced insulin secretion process. To our knowledge, this is the first time that results, presented in the current work, state that there is a positive effect produced by exercise on the correlation between insulin transduction in β-cells and insulin secretion. We conclude that obesity has harmful effects on the IRs of pancreatic islets by reducing their tyrosine phosphorylation, and exercise training can modulate this effect. We suggest that further studies must be performed to clarify what mechanisms in pancreatic β-cells are underlying the insulin deregulation in

obesity and how the exercise training effects are involved with these mechanisms.

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