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Changes in calcium fluxes in mitochondria, microsomes, and plasma membrane vesicles of livers from monosodium L-glutamate–obese rats

Monique Cristine de Oliveira[a], *Rosana Torrezan*[b], *Cecília Edna Mareze da Costa*[b], *Célia Regina Ambiel*[b], *Rodrigo Polimeni Constantin*[c], *Emy Luiza Ishii-Iwamoto*[a], *Clairce Luzia Salgueiro-Pagadigorria*[b,*]

[a] *Laboratory of Biological Oxidations, Department of Biochemistry, University of Maringá, 87020900 Maringá, Brazil*
[b] *Department of Physiological Sciences, University of Maringá, 87020900 Maringá, Brasil*
[c] *Laboratory of Liver Metabolism, Department of Biochemistry, University of Maringá, 87020900 Maringá, Brazil*

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ABSTRACT

The purpose of this work was to evaluate if the fat liver accumulation interferes with intracellular calcium fluxes and the liver glycogenolytic response to a calcium-mobilizing α_1-adrenergic agonist, phenylephrine. The animal model of monosodium L-glutamate (MSG)–induced obesity was used. The adult rats develop obesity and steatosis. Calcium fluxes were evaluated through measuring the $^{45}Ca^{2+}$ uptake by liver microsomes, inside-out plasma membrane, and mitochondria. In the liver, assessments were performed on the calcium-dependent glycogenolytic response to phenylephrine and the glycogen contents. The Ca^{2+} uptake by microsomes and plasma membrane vesicles was reduced in livers from obese rats as a result of reduction in the Ca^{2+}-ATPase activities. In addition, the plasma membrane Na^+/K^+-ATPase was reduced. All these matched effects could contribute to elevated resting intracellular calcium levels in the hepatocytes. Livers from obese rats, albeit smaller and with similar glycogen contents to those of control rats, released higher amounts of glucose in response to phenylephrine infusion, which corroborates these observations. Mitochondria from obese rats exhibited a higher capacity of retaining calcium, a phenomenon that could be attributed to a minor susceptibility of the mitochondrial permeability transition pore opening.

Author contributions: Monique Cristine de Oliveira conducted all experimental protocols, with initial orientation and support of the others authors. In the procedure of obesity induction, she had the support of Cecília Edna Mareze da Costa and in the experiments of liver perfusion, Rosana Torrezan. Rodrigo Polimeni Constantin and Clairce Luzia Salgueiro-Pagadigorria accompanied the assays involving isolation and purification of cellular fractions and also the subsequent experiments of calcium uptake using standard procedures. Célia Regina Ambiel da Silva contributed in the mitochondrial experiments and performed the liver glycogen measurement. Data interpretation and manuscript writing were done mainly with the efforts of Clairce Luzia Salgueiro-Pagadigorria and Emy Luiza Ishii-Iwamoto.

The research article was made with the participation and permission of all authors. Moreover, all experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá.

* *Corresponding author*. Tel.: +55 44 32614896; fax: +55 44 32614896.
E-mail address: clspagadigorria@uem.br (C.L. Salgueiro-Pagadigorria).

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1. Introduction

A critical complication of the metabolic syndrome is nonalcoholic fatty liver disease (NAFLD) [1-3]. In most cases, NAFLD is a benign condition characterized by simple and largely reversible steatosis. However, the steatotic liver becomes vulnerable to secondary insults and this appears to contribute to the occasional progression of NAFLD to more severe liver diseases [4-6].

Day and James [7] have proposed the "two-hit hypothesis" to explain the development of hepatic steatosis and the progression to inflammation (NASH), fibrosis, and cirrhosis. This hypothesis states that factors such as insulin resistance and impaired mitochondrial fatty acid oxidation contribute to development and progression of NAFLD. In fact, accumulating evidences suggest a major role of mitochondria dysfunction in fatty liver, independent of the etiology [8]. Mitochondrial dysfunction not only impairs fat homeostasis in the liver but also leads to an overproduction of reactive oxygen species (ROS) [9] and cell death [10].

It is known that increases in the intracellular pools of triglycerides and fatty acids are associated with oxidative stress [11,12], which could disturb intracellular calcium redistribution; dysregulation of Ca^{2+} homeostasis could, in turn, contribute to the pathogenesis and progression of NAFLD [10,13,14].

Intracellular calcium homeostasis depends on the specific transport systems located in the plasma and endoplasmic reticulum (ER) membranes as well in the inner mitochondrial membrane. The integrated actuation of these transport systems maintains the resting cytosolic calcium concentrations around 0.1 to 0.2 μmol/L. These low free cytosolic calcium concentrations are essential for the role of calcium as second messenger and for the maintenance of the cellular integrity [13-15].

Besides, we have amply demonstrated that drugs [16,17] or some pathological conditions [18] that are associated with abnormal intracellular calcium redistribution lead to metabolic alterations [19-22] and abnormal responses to Ca^{2+}-mobilizing hormones [23,24].

In this way, the purpose of this work was to evaluate if calcium transport by isolated microsomes, plasma membrane vesicles (PMVs), and mitochondria is altered in livers of animals with NAFLD. Livers from monosodium L-glutamate (MSG)–treated rats—a well-established model of hypophagic [25-27], hypothalamic obesity—were used in this study [28].

Monosodium L-glutamate–obese adult rats share several features that are common in human metabolic syndrome, including dyslipidemia, glucose intolerance, hyperinsulinemia, and decreased sensitivity to insulin [27,29,30]. Above all, adult animals develop extensive adiposity visceral [26,27] without the necessity of extreme dietary manipulations, such as methionine- and choline-deficient diets.

To evaluate whether the changes in calcium transport revealed in separated subcellular fractions result in impaired calcium signaling in intact liver, we also evaluated the glycogenolytic response of livers from obese rats to phenylephrine—an α_1-adrenergic agonist that changes calcium fluxes by means of known mechanisms [31]—and the liver glycogen contents.

2. Methods

2.1. Material

Adenosine 5′-triphosphate (ATP), adenosine 5′-monophosphate, phenylmethylsulfonyl fluoride, and substrates were purchased from Sigma Chemical (St Louis, MO). $^{45}CaCl_2$ was purchased from NEN Life Science Products (Boston, MA). Cyclosporin A (CsA) was provided by Dr William S Taguchi (Maringá, Brazil). All other reagents were from the best available grade. Liver perfusion apparatus was built in the workshops of University of Maringa.

2.2. Animals and MSG treatment

Male newborn Wistar rats received subcutaneous injections of MSG (4 g/kg body weight [BW] per day) or hyperosmotic saline solution (1.25 g/kg BW per day) during the first 5 days of life [28]. Control and MSG-obese rats were fed with a standard

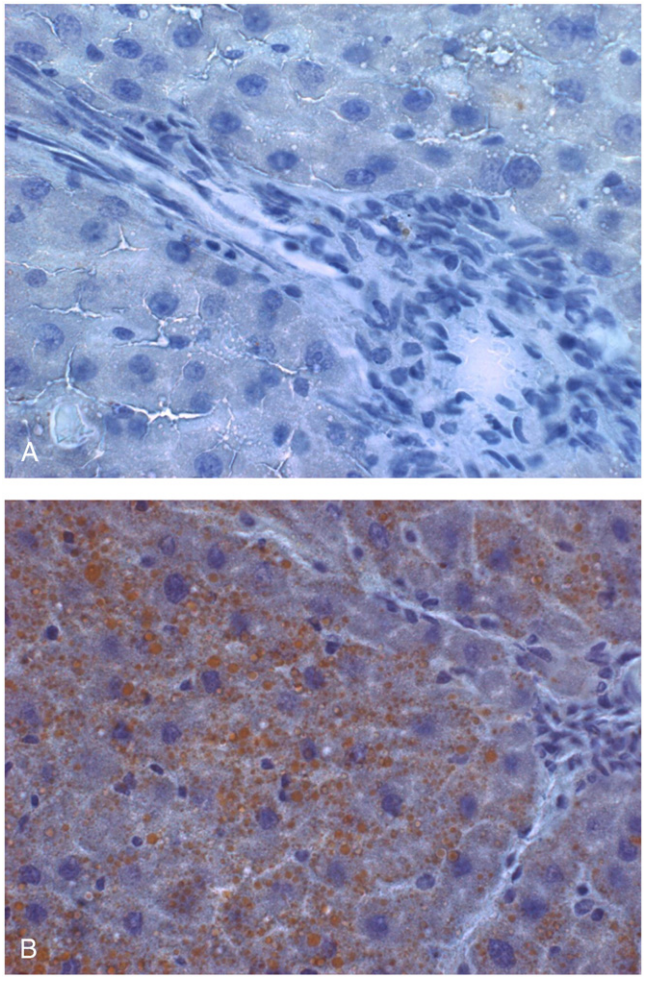

Fig. 1 – Liver histochemical analyses. Liver fragments were frozen at –20°C, sectioned by cryostat, and stained for lipids using Sudan III. Panels A (control) and B (MSG-obese) were captured at 40×. Contrasting with control rats, livers from MSG-obese rats presented considerable amounts of lipid inclusions (in orange).

rodent diet. Batches of 110- to 140-day-old rats were weighed, and the Lee index was used as a predictor of obesity [32]. In the procedure of liver removal or perfusion, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and euthanized afterward. Liver histochemical analysis were conducted to prove the existence of steatosis [33] (Fig. 1). All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá.

2.3. Separation of subcellular fractions

Mitochondria and microsomes were isolated from liver homogenates of control and obese rats by differential centrifugation [34]. Liver PMVs were isolated from the postmitochondrial supernatant as described by Armstrong and Julie [35] with some modifications [17]. Protein contents of subcellular fractions were measured using bovine serum albumin as standard [36].

2.4. Plasma membrane and microsomal vesicles characterization

The final plasma membrane and microsomal vesicles preparations were assayed for 5′-nucleotidase [37], glucose-6-phosphatase [38] and Mg^{2+}-ATPases [39]. The latter were assayed in the presence of 10 μg/mL oligomycin. Besides, microsomal membrane integrity was evaluated by the latency of the mannose-6-phosphatase activity [40]. The activities of these enzymes were determined by the amount of phosphate existing in the supernatant [41].

2.5. Microsomal calcium transport

The Mg^{2+}ATP-dependent microsomal $^{45}Ca^{2+}$ uptake was determined using 5 mmol/L ammonium oxalate [42] or 5 mmol/L glucose-6-phosphate as trapping agents of calcium [43]. Microsomes (0.24-0.36 mg/mL) were incubated at 37°C in 1.0 mL of a medium containing 0.1 mol/L KCl, 0.02 mol/L Tris (pH 7.4), 5 mmol/L $MgCl_2$, 20 μmol/L $CaCl_2$, and 0.015 μCi/mL $^{45}CaCl_2$. Aliquots of 100 μL were removed as a function of time, and microsomes were separated, at appropriate times, by vacuum filtration on 13-mm–diameter cellulose acetate filters (Millipore Corp, Billerica, MA; 0.2 μm pore diameter). The $^{45}Ca^{2+}$ retained on the filter was determined by liquid scintillation spectrometry. Uptake of $^{45}Ca^{2+}$ was initiated by the addition of 5 mmol/L ATP and expressed as nanomoles per milligram of microsomal protein.

2.6. Calcium uptake by liver PMVs

The ATP-dependent $^{45}Ca^{2+}$ uptake by inside-out PMVs (0.1-0.2 mg/mL) was measured at 37°C in a medium containing 0.1 mol/L KCl, 0.02 mol/L Tris (pH 7.4), 5 mmol/L $MgCl_2$, 1.0 mmol/L ATP, 20 μmol/L $CaCl_2$, and 0.015 μCi/mL $^{45}CaCl_2$ [44]. The reaction was initiated by ATP addition and stopped at appropriate time intervals by vol/vol dilution of 200 μL-aliquots with a cold medium containing 0.1 mol/L KCl, 0.02 mol/L Tris (pH 7.4), and 1.0 mmol/L $LaCl_3$. The samples were quickly centrifuged for 5 minutes at 13 400*g*, and $^{45}Ca^{2+}$ existing in the supernatant was counted by liquid scintillation spectrometry. Na^+-sensitive Ca^{2+} uptake was assessed in the same manner, except that the 100 mmol/L KCl in the incubation medium was replaced by 50 mmol/L KCl plus 50 mmol/L NaCl, in the absence or in the presence of 0.5 mmol/L ouabain. $^{45}Ca^{2+}$ uptake was expressed as nanomoles per milligram protein.

2.7. Mitochondrial calcium uptake by mitochondria stimulated with ATP or succinate

Electrophoretic mitochondrial $^{45}Ca^{2+}$ uptake was measured in the presence of 2 μmol/L rotenone. Mitochondria (0.15-0.25 mg/mL) were incubated at 37°C in a specific medium [45] in the presence of $CaCl_2$ (1.0 or 20 μmol/L) and 0.015 μCi/mL $^{45}CaCl_2$ and in the absence or presence of 1.0 μmol/L CsA. The reaction was started by the addition of 1.0 mmol/L succinate [45] or 5.0 mmol/L ATP [46] and stopped at appropriate time intervals by vol/vol dilution with the same cold medium, without calcium but containing 1.0 mmol/L $LaCl_3$. $^{45}Ca^{2+}$ was counted in 100-μL aliquots of 13 400*g* supernatants by liquid scintillation spectrometry.

2.8. Liver perfusion experiments

A hemoglobin-free nonrecirculating system of in situ liver perfusion of fed rats was used in this work to measure the phenylephrine-induced glucose release. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) saturated with an oxygen/carbogen dioxide mixture (95%/5%). The fluid was pumped through a temperature-regulated membrane oxygenator (37°C) before entering the liver via a cannula inserted in the portal vein. After a 10-minute preperfusion period, phenylephrine (2 μmol/L) was infused during 20 minutes. Samples of the effluent perfusion fluid were collected at 2-minute intervals. D-Glucose was measured by the method of glucose oxidase [47] and referred to the wet weight of the liver.

Table 1 – Plasma membrane and microsomal vesicles characterization

Enzymes	Microsomal vesicles	
	Control	Obese
Glucose-6-phosphatase	997.00 ± 107.00	502.50 ± 44.18*
5′-Nucleotidase	91.82 ± 10.94	52.78 ± 9.19*
Mannose-6-phosphatase	35.90 ± 10.04	37.36 ± 8.17
Mg^{2+}-ATPase	169.80 ± 21.12	76.43 ± 7.43*
Enzymes	**PMVs**	
	Control	Obese
Glucose-6-phosphatase	75.50 ± 6.55	61.15 ± 4.96
5′-Nucleotidase	1082.00 ± 12.30	668.40 ± 75.99*
Mg^{2+}-ATPase	1092.00 ± 81.73	645.80 ± 74.90*

The activities of the following enzymes were determined by the amount of phosphate released in the medium and expressed as nanomoles per minute per milligram protein. Data are the means ± SEM of 3 to 9 experiments with identical protocol. Statistical significance relative to the controls is indicated by asterisks.

* $P < .05$, by means of Student *t* test.

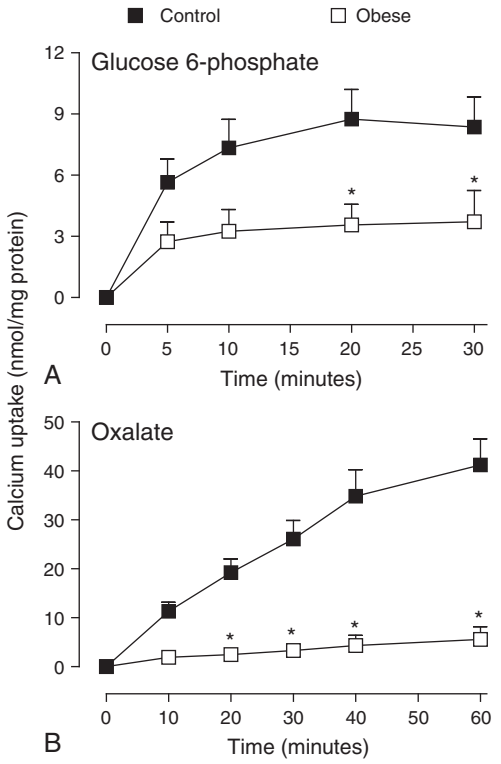

Fig. 2 – Time courses of Mg^{2+}ATP-dependent calcium uptake by rat liver microsomes from control and MSG-obese rats in the presence of glucose-6-phosphate (A) or oxalate (B). The microsomal fractions (0.24-0.36 mg/mL) were incubated at 37°C in a medium containing 0.1 mol/L KCl, 5 mmol/L $MgCl_2$, 20 μmol/L $CaCl_2$, 0.2 μCi/mL $^{45}CaCl_2$, and 0.02 mol/L Tris (pH 6.8 or 7.2). The reaction was initiated by the addition of 5.0 mmol/L ATP; and at indicated times, 100-μL samples were filtered through Millipore filters, and the $^{45}Ca^{2+}$ content was counted by liquid scintillation spectrometry. A, Calcium uptake by microsomes from control (■) and MSG-obese (□) rats in the presence of 0.5 mmol/L glucose-6-phosphate as trapping agent. B, Calcium uptake by microsomes from control (■) and MSG-obese (□) rats using 5 mmol/L ammonium oxalate as trapping agent. The ATP-independent calcium $^{45}Ca^{2+}$ uptake and the unspecific binding of calcium to the filters were subtracted from the energy-dependent uptake (values just before ATP addition). Each point represents the mean ± SEM of 4 to 6 experiments.

2.9. *Liver glycogen contents*

Glycogen contents were determined in freshly isolated livers. Portions of approximately 2 g were freeze-clamped with liquid nitrogen. These samples were homogenized and extracted with 10 mL of 6% $HClO_4$. The supernatant was neutralized with 5.0 N K_2CO_3 and used for the enzymatic glycogen assay [48]. The amount of glycogen was expressed as micromoles of glucose per gram of liver.

2.10. *Statistical analysis*

The statistical significance of the differences between parameters was evaluated by means of Student *t* test or 2-way analysis of variance. The latter was applied after submitting the data to variance analysis. The results are mentioned in the text as the *P* values; $P < .05$ was adopted as a criterion of significance. The glycogen catabolism was evaluated by means of the area under curve (AUC) and expressed as micromoles per gram of liver.

3. Results

3.1. *Characterization of the subcellular preparations*

To evaluate if the plasma membrane and microsomal preparations obtained from control and obese rats presented

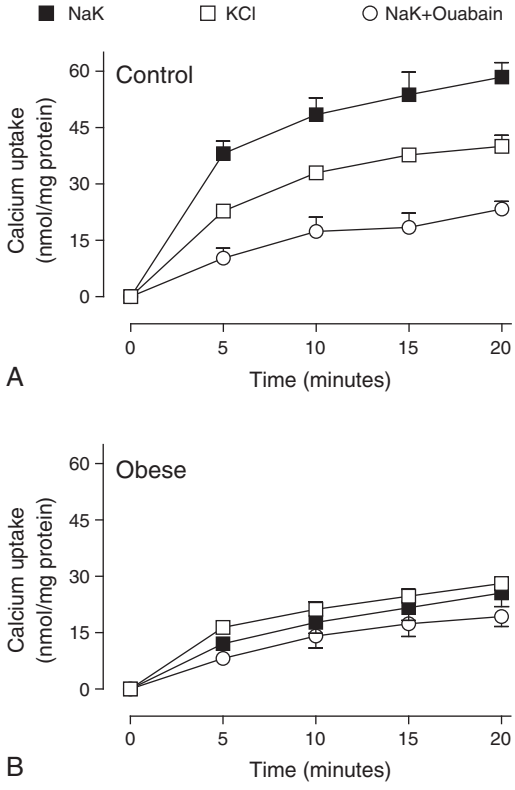

Fig. 3 – Time courses of Mg^{2+}ATP-dependent calcium uptake by inside-out rat liver PMVs from control and MSG-obese rats. A, $^{45}Ca^{2+}$ uptake by PMVs from control rats was measured in the medium of 100 mmol/L KCl (□) and 50 mmol/L NaCl plus 50 mmol/L KCl in the absence (■) or in the presence (○) of 0.5 mmol/L ouabain. B, $^{45}Ca^{2+}$ uptake by PMVs from MSG-obese rats incubated in the same media as above. The PMVs (0.1-0.2 mg/mL) were incubated at 37°C in each specific medium, and the reaction was initiated by the addition of ATP and stopped at the indicated times by transferring 200-μL aliquots to an equal volume of a cold medium containing 0.1 mol/L KCl, 0.02 mol/L Tris (pH 7.4), and 1.0 mmol/L $LaCl_3$. After centrifugation, the radioactivity of the supernatant was counted by liquid scintillation spectrometry. The ATP-dependent $^{45}Ca^{2+}$ uptake was calculated by the difference between the $^{45}Ca^{2+}$ existing in the supernatant just before ATP addition and that one after the addition of ATP. The data points represent the mean ± SEM of 4 to 8 experiments. *$P < .05$.

the same degree of purity, these fractions were assayed through the measurement of glucose-6-phosphatase and 5′-nucleotidase activities. Table 1 shows that the microsomal preparations obtained from livers of control rats presented high purity [42]. Microsomes from obese rats exhibited lower glucose-6-phosphatase (–49.6%) and 5′-nucleotidase (–42.5%) activities. The average 6-phosphatase to 5′-nucleotidase ratio, however, remained similar to that of control rats (>9.0). The test of accessibility for mannose-6-phosphate revealed that liver microsomes from control rats also presented a high degree of integrity, with glucose-6-phosphatase to mannose-6-phosphatase ratio of about 27.77 [40]. Microsomes from obese rats, however, presented a lower glucose-6-phosphatase to mannose-6-phosphatase ratio of about 13.45. In addition, Table 1 shows that the total Mg^{2+}-ATPase activities were decreased in microsomes from obese rats in about 60%.

The enzymatic characterization of PMVs is also shown in Table 1. As can be seen, PMVs from obese rats presented lower 5′-nucleotidase and glucose-6-phosphate activities (–38.3% and –19.0%, respectively) than those from control rats. In both cases, however, the high values of the 5′-nucleotidase to glucose-6-phosphatase ratio (>10) revealed that the contamination of these fractions by microsomes was minimal [44]. In addition, the PMV Mg^{2+}-ATPase activities were about 40.9% lower in obese than in control rats.

3.2. *ATP-dependent calcium uptake by liver microsomes*

The MSG-obese rats developed extensive steatosis—as could be demonstrated by liver histochemical analyses (Fig. 1)—and, at the end of experimental period, presented higher body weights (405.91 ± 9.1 g) than control rats (325.86 ± 6.06 g), although their average daily food consumption (19.44 ± 1.01 g) was lower than control rats (27.27 ± 0.99 g).

Based on previous observations that liver calcium fluxes [19-22] as well as the responses to calcium-mobilizing-hormones [23,24] were altered in several pathological conditions, the initial experiments were planned to evaluate the effects of fat liver accumulation on specific transport systems involved in the cellular calcium homeostasis in livers from MSG-treated rats.

As shown in Fig. 2A, in the presence of 0.5 mmol/L glucose-6-phosphate as trapping agent [41], accumulation of $^{45}Ca^{2+}$ was fast initially, but tended to reach an apparent equilibrium at 30 minutes with a total accumulation of 8.36 ± 1.48 nmol/mg protein in microsomes from control rats. At this time, microsomes from obese rats accumulated significantly lower amounts of calcium (3.72 ± 1.53 nmol/mg protein).

In the presence of 5.0 mmol/L ammonium oxalate [43], net calcium uptake progressed linearly for a period of 60 minutes, as illustrated by Fig. 2B. Under these experimental conditions,

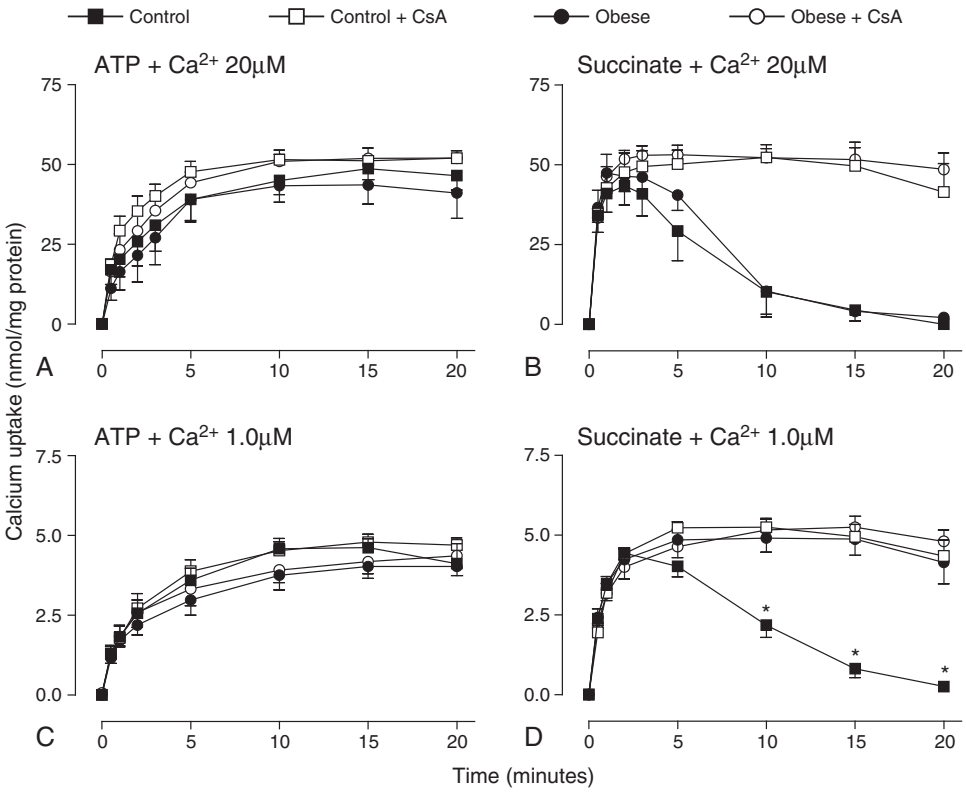

Fig. 4 – Calcium uptake by mitochondria stimulated with ATP or succinate. Mitochondria (0.15-0.25 mg/mL) from control were incubated at 37°C in a medium containing 0.125 mol/L sucrose, 0.065 mol/L KCl, 5 mmol/L $MgCl_2$, 0.2 mmol/L potassium phosphate, 3.0 mmol/L Hepes (pH 7.2), 2 µmol/L rotenone, 20 µmol/L (A and B) or 1.0 µmol/L $CaCl_2$ (C and D), and 0.015 µCi/mL $^{45}CaCl_2$. The reaction was initiated by the addition of 5.0 mmol/L ATP (A and C) or 1.0 mmol/L succinate (B and D) in the absence or presence of 1.0 µmol/L CsA. $^{45}Ca^{2+}$-uptake was calculated as the difference between the $^{45}Ca^{2+}$ existing in the supernatant immediately before and after the addition of ATP or succinate. Each data point represents the mean ± SEM of 5 or 6 (ATP or succinate series) experiments. *P < .05.

the calcium uptake reflects exclusively the Ca^{2+}-Mg^{2+}-ATPase activity. In the presence of oxalate, microsomes from livers of obese rats accumulated less calcium than microsomes from control rats. This difference was statistically significant from 20 minutes of incubation. After 60 minutes, microsomes of control rats accumulated 41.21 ± 5.318 nmol of Ca^{2+} per milligram protein, whereas microsomes from obese rats accumulated 5.571 ± 2.62 nmol per milligram protein.

3.3. *Mg^{2+}ATP-dependent calcium uptake in liver PMVs*

The ATP-dependent Ca^{2+} uptake by inside-out PMVs is shown in Fig. 3. The time courses of Ca^{2+} uptake by PMVs from control rats are presented in the panel A. Upon the ATP addition, calcium uptake proceeded in a linear fashion for 5 minutes; and after 20 minutes, a tendency toward a steady state was apparent with 100 mmol/L KCl medium. At this time, the total actively stored $^{45}Ca^{2+}$ was equal to 39.97 ± 2.97 nmol/mg protein. The PMVs from control rats accumulated higher amounts of calcium in the equiosmolar NaCl/KCl medium. At 20 minutes of incubation, calcium uptake reached an apparent equilibrium with a total accumulation of 58.43 ± 3.82 nmol/mg protein. The difference between the amount of calcium accumulated in the KCl medium and in the presence of 50 mmol/L NaCl plus 50 mmol/L KCl represents the Na^+-sensitive uptake, in other words, the Na^+/K^+-Mg^{2+}-ATPase, which pumps sodium inside the vesicles and causes the Na^+/Ca^{2+} exchanger to operate in the direction of sodium efflux and calcium influx. In the NaCl/KCl medium and in the presence of 0.5 mmol/L ouabain, the amount of stored calcium, after 20 minutes, was lower than that measured in the KCl medium (8.154 ± 1.589 nmol/mg protein).

Panel B of the Fig. 3 shows the ATP-dependent Ca^{2+} uptake by PMVs from obese rats. The amount of stored calcium was significantly lower than that of control rats in the KCl medium, and steady-state values of 28.06 ± 1.79 nmol/mg protein were reached after 20 minutes. Besides, there are no significant differences between the amounts of calcium accumulated in this medium and those in the NaCl/KCl medium in the absence or presence of 0.5 mmol/L ouabain, where the total actively stored $^{45}Ca^{2+}$ reached values of 25.52 ± 5.74 and 19.27 ± 2.61 nmol/mg protein in the subcellular fraction.

3.4. *Calcium uptake by mitochondria stimulated with ATP or succinate*

Fig. 4 shows the electrophoretic uptake of $^{45}Ca^{2+}$ over time, in the presence of 20 μmol/L Ca^{2+} (panels A and B) or 1.0 μmol/L calcium (panels C and D) and in mitochondria stimulated with ATP (panels A and C) or succinate (panels B and D). In the presence of 20 μmol/L calcium, there was no observable difference in calcium uptake between liver mitochondria from control and obese rats when stimulated with either succinate or ATP. The addition of ATP (panel A) caused a progressive increase in $^{45}Ca^{2+}$ uptake through the whole incubation period in mitochondria from both control and obese rats. The addition of 1.0 μmol/L CsA did not alter the Ca^{2+} uptake kinetics. In the presence of succinate (panel B) and 20 μmol/L calcium, mitochondria from control and obese rats exhibited a rapid accumulation of $^{45}Ca^{2+}$, which reached peak values at around 2 minutes. After this period, the amount of $^{45}Ca^{2+}$ progressively decreased, reaching negligible values after 20 minutes of incubation. This transience in the kinetics of calcium uptake can be attributed to the opening of the permeability transition pore (PTP), as we reported previously [16,17]. In fact, CsA (1 μmol/L) completely prevented the release of calcium from mitochondria and under these conditions, the Ca^{2+} uptake kinetics became similar to that observed when mitochondria were energized with ATP (with or without CsA).

In the presence of 1.0 μmol/L calcium (a more physiological concentration) and ATP as a stimulant (panel C), liver mitochondria from control and obese rats exhibited nearly identical uptake kinetics. With succinate (panel D), mitochondria from control rats exhibited the same calcium uptake profile as that observed in the presence of 20 μmol/L calcium; however, in mitochondria from obese rats, the amount of accumulated calcium remained elevated and was significantly higher in mitochondria from obese rats after 10 minutes of incubation.

3.5. *Phenylephrine-induced glycogenolysis in perfused liver and liver glycogen contents*

To evaluate glycogen catabolism, livers from well-fed control and obese rats were perfused with substrate-free perfusion fluid. Under these conditions, the rate of glucose release provides a good approximation for glycogenolysis [49]. Fig. 5 shows the time courses of glucose release from livers of control and obese rats. Before the phenylephrine infusion, livers from control and obese rats released similar and small amounts of glucose (basal values). The infusion of 2 μmol/L

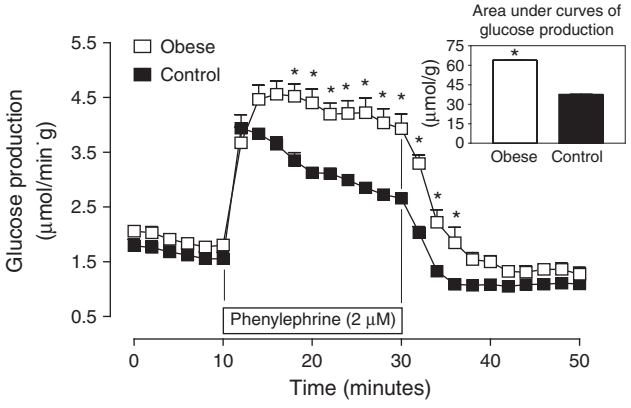

Fig. 5 – Effect of phenylephrine (2 μmol/L) on hepatic glucose production. Livers from fed control (■) or MSG-obese (□) rats were perfused as described in "Methods." Glucose was assayed by glucose oxidase method. The glucose release was calculated from the arteriovenous concentrations differences and expressed as micromoles per minute per gram of liver. Each point of the curve represents the mean ± SEM of 8 animals for both groups (*P < .05, Student t test). The total amounts of glucose released calculated as the AUCs ± SEM as obtained from these perfusion experiments are presented in the upper right corner (*P < .05, 2-way analysis of variance [ANOVA]).

phenylephrine promoted a rapid and transient increase in glycogenolysis in both groups. However, this increase was significantly greater and more prolonged in the obese rats. Peak values were attained in less than 5 minutes after the beginning of phenylephrine infusion. In the upper right corner of Fig. 5, the amounts of glucose release that was calculated by the AUC are presented. The amounts of glucose released by livers from obese rats (63.99 μmol/g) were 41% higher than those found in control rats (37.57 μmol/g) ($P < .05$), although the livers from MSG-obese rats were significantly smaller (7.11 ± 0.19 g) than the livers from control rats (11.41 ± 0.43 g) and exhibited similar glycogen contents (217.33 ± 21.86 μg/g of liver) of control rats (207.70 ± 18.86 μg/g of liver).

4. Discussion

Liver microsomes from obese rats exhibited a lower capacity of retaining calcium, independently of the calcium trapping agent used, evidencing a reduction in the hydrolytic capacity of the Ca^{2+}-ATPase. This observation is corroborated by the lower Mg^{2+}-ATPases activities found in the microsomes from obese rats.

In isolated hepatocytes, this Ca^{2+}-ATPase—dependent on magnesium, calcium, and calmodulin [15]—is also indirectly stimulated by glucose-6-phosphate, which provides intravesicular phosphate anions as a result of the enzymatic hydrolysis by glucose-6-phosphatase [43]. In fact, it is believed that the location of glucose-6-phosphatase in the luminal surface of the ER represents a physiological adaptation, by which increasing of glucose-6-phosphate levels—as a product of glycogenolysis stimulated by Ca^{2+}-mobilizing hormones—ameliorates the calcium reuptake by the ER [40,43].

Another factor contributing to the lower liver microsomal Ca^{2+}-ATPase activity of obese rats should be alterations in the fatty acid composition of membrane [50]. In fact, the tests of mannose-6-phosphate accessibility revealed that the integrity of the ER membrane from obese rats was altered.

Inside-out PMVs from obese rats also exhibited a lower calcium uptake capacity. Reduced membrane Ca^{2+}-ATPase activity has been reported in various tissues of hypothalamic obesity [51] and obese *fa/fa* rats [52]. In humans, decreased erythrocyte Ca^{2+}-ATPase activities associated with increased insulin plasma levels [53] and negatively related to body mass index [54] have been reported. Both these features are found in the MSG-induced obesity [29,30].

Furthermore, PMVs from obese rats did not exhibit increments in the amounts of calcium accumulated in the presence of sodium, suggesting a lower Na^+-K^+-ATPase activity. In fact, the total Mg^{2+}-ATPases activities of plasma membrane, as measured by the amount of phosphate released, were reduced in obese rats.

In intact cells, a reduced capacity of ER in retaining calcium associated with reduced plasma membrane Na^+-K^+-ATPase and Ca^{2+}-ATPase activities should lead to increased resting calcium levels in the obese rats. This hypothesis was evidenced indirectly by measuring the glycogenolytic effect of phenylephrine.

In perfused livers, the stimulation of glycogenolysis by α-adrenergic agonists depends on the calcium redistribution, which follows a strict temporal sequence: during the first 1 to 2 minutes of the α-agonist action, calcium is mobilized from intracellular nonmitochondrial stores—preferentially the ER—that is, at least partially, extruded. Afterward, a higher steady-state rate of calcium cyclization through the plasma membrane sustains the high intracellular levels and the response to the α-agonist [31]. This cyclization appears to involve calcium extrusion, mainly by the membrane Ca^{2+}-ATPase and calcium influx via channels activated by the binding of the agonist to the receptor (receptor-operated Ca^{2+} channel) [55]. The withdrawal of the α-agonist causes a rapid reuptake of calcium from extracellular fluid through the activation of store-operated Ca^{2+} channels [31,55].

The responses of the perfused livers from obese rats to phenylephrine were consistent with these observations. The transient response to the agonist, which depends on the intracellular stores mobilization, was unaffected. Otherwise, the sustained response was increased in obese rats, probably as a result of a lower rate of calcium extrusion by the plasma membrane Ca^{2+}-ATPase and lower rate of calcium uptake into the ER. With the removal of the agonist, the return to basal conditions was slower in livers from obese rats, a finding that could be the result of a minor rate of calcium uptake into the ER.

It should be emphasized that these differences in glucose release cannot be attributable to differences in the glycogen contents because similar content values were found in livers from both control and obese rats. Besides, livers from MSG-obese rats were smaller than those from control. It seems therefore plausible to suppose that the higher sustained rate of glycogenolysis could be, at least in part, the result of higher resting intracellular calcium levels existing in hepatocytes from obese rats.

Besides, increased intracellular calcium levels could be a factor that contributes to fat liver accumulation. In adipocytes of obese humans, increased intracellular calcium levels are associated with increased fatty acid synthase activity; and this contributes to development of obesity [56]. Moreover, it was demonstrated that, in adipocytes, elevation in cytosolic calcium is one of the factors triggering the insulin resistance in obesity [57].

The perfusion experiments also allowed verifying that the minor glucose-6-phosphatase activity found in liver microsomes of obese rats did not reduce the liver glucose release. This is in agreement with earlier reports that demonstrated that an inhibition on glucose efflux due to a decrease in glucose-6-phosphate activity occurs only when the latter is strongly affected [58]. This is partly due to the high activity of the enzyme and to the fact that it catalyzes the terminal step in the process of glucose release from endogenous glycogen. Moderate reduction in the glucose-6-phosphatase activity, as observed in the present work, simply increases the substrate concentration to higher steady-state values, reestablishing the original flux [58].

A question that deserves a comment is about the changes observed in calcium uptake by isolated mitochondria. Mitochondria are organelles of high capacity and low affinity for calcium uptake [15]. Mitochondrial Ca^{2+} overload, however, exerts a deleterious effect over mitochondria by causing the PTP opening, initiating the complex sequence of the events that culminates in the cell death [10].

Contrasting with microsomes and PMVs, mitochondria from obese rats exhibited an enhanced retention of calcium in the presence of 1.0 μmol/L calcium and succinate as a stimulant. This higher capacity of calcium retention by mitochondria from obese rats could be the result of a minor susceptibility to the PTP opening [16-18].

A higher capacity of retaining calcium was already described for both brown adipose tissue and liver mitochondria from *ob/ob* mice [59]. One possibility is that liver mitochondria from MSG-treated rats undergo an enlargement in mitochondrial matrix volume, as described for liver mitochondria of obese *ob/ob* mice [60]. This would help to delay the opening of the PTP.

Therefore, it seems that, with respect to calcium-induced PTP opening, mitochondria of obese rats exhibit a reduced propensity for initiating the events leading to cell death.

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