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ORIGINAL ARTICLE

Diet-induced obesity causes hypothalamic neurochemistry alterations in Swiss mice

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Abstract

The aim of this study was to assess inflammatory parameters, oxidative stress and energy metabolism in the hypothalamus of diet-induced obese mice. Male Swiss mice were divided into two study groups: control group and obese group. The animals in the control group were fed a diet with adequate amounts of macronutrients (normal-lipid diet), whereas the animals in the obese group were fed a high-fat diet to induce obesity. Obesity induction lasted 10 weeks, at the end of this period the disease model was validated in animals. The animals in the obese group had higher calorie consumption, higher body weight and higher weight of mesenteric fat compared to control group. Obesity showed an increase in levels of interleukin 1β and decreased levels of interleukin 10 in the hypothalamus. Furthermore, increased lipid peroxidation and protein carbonylation, and decreased level of glutathione in the hypothalamus of obese animals. However, there was no statistically significant difference in the activity of antioxidant enzymes, superoxide dismutase and catalase. The obese group had lower activity of complex I, II and IV of the mitochondrial respiratory chain, as well as lower activity of creatine kinase in the hypothalamus as compared to the control group. Thus, the results from this study showed changes in inflammatory markers, and dysregulation of metabolic enzymes in the pathophysiology of obesity.

Keywords Obesity · Hypothalamus · Inflammation · Oxidative stress · Energy metabolism

Introduction

The World Health Organization (2015) defines obesity as a chronic disease, being the main characteristic an excessive accumulation of fat in adipose tissues. The disease has a multifactorial origin and is related to the development of numerous co-morbidities, including cardiovascular disease, type 2 diabetes mellitus (DM2), dyslipidemia, lung diseases, osteoarticular diseases, some types of cancer, and psychosocial disorders (Knight 2011).

Recent data indicate that the percentage of obese or overweight individuals is increasing, and concomitantly, the morbidity and mortality rates (WHO 2015), which compromises the quality of life and longevity of the population (Segula 2014). In addition, expenditures on obesity-related treatments are progressive and have a strong impact on the economy (Bahia and Araújo 2014; Tigbe et al. 2013). Thus, obesity is described as a public health problem (Serra-Majem and Bautista-Castaño 2013; WHO 2015).

Despite the importance of the studies on obesity, the pathophysiology of this disease still requires clarification (Thaler et al. 2013). Data indicate that obesity progresses in both the peripheral tissues (Ferrante Jr 2013; Lehr et al. 2012), and in the central nervous system (CNS) (Ferrante 2013). However, it is not completely clear which of these tissues is the first to contribute to the excessive fat accumulation in the adipose tissue (Thaler et al. 2013).

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Fat accumulation leads to substantial changes in the adipose tissue, including the dysregulation of adipokine secretion (Lehr et al. 2012) and an infiltration and activation of macrophages (Ferrante 2013). As a result, there is an increased expression of proinflammatory cytokines and reactive oxygen species (ROS) by infiltrating macrophages (Lumeng et al. 2007). Studies have shown that obesity progresses in parallel with mitochondrial alternations in peripheral tissues such as excessive mitochondrial ROS production (Matsuda and Shimomura 2013).

With regard to the CNS, research shows that the impairment of target structures, such as the hypothalamus (Hochberg and Hochberg 2010; Thaler et al. 2012; Velloso and Schwartz 2011), may lead to obesity by reducing the performance of these structures, contributing to excessive body-fat accumulation (McNay et al. 2012; Milanski et al. 2009). The pathophysiology of obesity includes loss of sensitivity to afferent peripheral humoral signals, such as leptin, on one hand, and dysfunctional afferent signals, on the other hand (Milanski et al. 2009). The most important afferent signals deranged are energy regulation by the sympathetic nervous system and regulation of insulin secretion (Hochberg and Hochberg 2010). According to Kennedy et al. (2009), energy stored in adipose tissue is communicated to the hypothalamus through circulating signals that ultimately serve to adjust food intake in response to variation in energy depot size.

Saturated fatty acids can activate specific toll-like receptors in the hypothalamus and, therefore, induce an inflammatory process in that site (Milanski et al. 2009). Activation of the microglia plays an important role in the release of proinflammatory cytokines such as Tumor Necrosis Factor -Alpha (TNFα), Interleulin 1β (IL-1β) and Interleukin 6 (IL-6) (Gao et al. 2014; Loane and Byrnes 2010) and ROS (Cooney et al. 2013), culminating in cell damage (Dheen et al. 2007; Nakajima and Kohsaka 2001). Loss of integrity of the blood-brain barrier (BBB) may influence obesity progression, given that immune cells of the peripheral tissues may be recruited to the CNS and contribute to the inflammatory process in the hypothalamus (Buckman et al. 2014).

The brain has a high energy demand to perform its various functions (Cheng and Ristow 2013). Therefore, adequate supply of adenosine triphosphate (ATP) by mitochondria is vital for proper cell performance (Nelson and Cox 2011). Likewise, the antioxidant defense in the CNS has a very important role, since mitochondria is the major producer of ROS, which can cause oxidative damage (Federico et al. 2012).

Many studies use high-fat diets to induce obesity in rodents (Kennedy et al. 2009). In order to investigate the influence of obesity on inflammatory and biochemical parameters in the hypothalamus of mice, the present study used an animal model of obesity based on the consumption of a high-fat diet. Wang et al. (2012) investigated hypothalamic inflammation in diet-induced obesity and obesity-resistant rats, and identified that the consumption of saturated chain lipids is related to excess adiposity, changes in inflammatory markers, and dysregulation of metabolic enzymes. However, mitochondrial function and oxidative damage have not been studied (Kennedy et al. 2009).

Taken together, these data reinforce the need for a better understanding of the effect of obesity on the hypothalamus, given that this structure plays a central role in controlling energy balance (Begg and Woods, 2013). The aim of this study was to assess inflammatory parameters, oxidative stress parameters, and energy metabolism in the hypothalamus of diet-induced obese mice.

Materials and methods

Animals

Thirty male Swiss mice (*Mus musculus*), 40 days old, weighing 30-40 g, from the vivarium of the Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil. Animals were housed individually in cages and were kept in a climatized environment with controlled light cycle of 12:12 h (7 h–19 h) and temperature of 23 ± 3 °C. All experiments were approved by the Ethics Committee on Animal Use of the University of Southern Santa Catarina (Protocols: 15.002.4.01.IV., 15.001.4.01.IV., and 15.005.4.01.IV).

Obesity induction

The protocol for obesity induction and diet composition was based on previous studies (Cintra et al. 2012; Razolli et al. 2015). The control group was fed a standard normal-lipid diet ($n = 15$), whereas the obese group received a high-fat diet (n = 15). The sample of size n = 15 was large enough to present a good homogeneity between samples for analyses performed in this study. Five different animals from each of the experimental groups ($n = 5$ inflammatory parameters, n = 5 oxidative stress and n = 5 respiratory chain) were used for each analysis. All animals had free access to food and water. Food consumption was measured daily, and at the end of the 10-week period, obesity was confirmed by assessing body weight and weight of visceral fat.

Diets

Mice standard diet (purified low-fat diet) and obesity induction diet (purified high-fat diet) were purchased from PragSolutions Biosciences, a company specialized in production of standardized diets for animal experimentation. The diet composition was based on a previous study conducted by Cintra et al. (2012). The high-fat diet provided more calories

and a higher percentage of saturated fat than the standard diet. Diet composition, energy content, and percentage of macronutrients are described in Table 1. The calculations were based on the information provided by the manufacturer.

Validation for animal model of obesity

Assessment of food intake was performed based upon the difference between the amount of food offered to the animal and the amount of food remaining in the cage after 24 h. These data were expressed as calories consumed, taking into account the number of calories of each diet (normal-lipid diet = 3798 Kcal/kg; high-fat diet = 5358 Kcal/kg) as shown in Table 1. Body weight of the animals were monitored throughout the diet-induction period. The mesurements were made once a week, using an electronic digital scale. After the death of the animals, the abdominal cavity was opened, and the mesenteric, epididymal, and retroperitoneal adipose tissues was dissected out. The samples were cleaned and weighed using a high-precision analytical balance. The total weight of the three fats provided the total amount of visceral fat, as previously described by Hansen et al. (1997).

Cytokine levels in the hypothalamus

The brain was rapidly removed, and the hypothalamus was collected and stored at −80 °C. Concentrations of IL-1β and IL-10 were determined in the hypothalamus by enzyme-linked immunosorbent assay (ELISA) on microplate reader using commercial kits (Peprotech, Sao Paulo, Brazil). The results were expressed as picograms per milligram of total protein (pg / mg protein).

Table 1 Composition and caloric value of normal-lipid and high-fat diets, at every 1000 g. Percentage of calories from the macronutrients used

	Normal-lipid diet		High-fat diet	
Macronutrients	% Kcal		% Kcal	
Carbohydrates	69%		26%	
Proteins	21%		15%	
Lipids	10%		59%	
Ingredients	g/kg	kcal/kg	g/kg	kcal/kg
Maize starch	427.5	1710	115.5	462
Casein	200	800	200	800
Saccharose	132	528	132	528
Dextrinized starch	100	400	100	400
Soy oil	40	360	40	360
Lard	–	–	312	2808
Cellulose	50	–	50	–
Mineral mix	35	–	35	–
Vitamines mix	10	–	10	–
L-Cystine	3	–	3	–
Choline bitartrate	2.5	–	2.5	–
Butyl hydroxytoluene	0.028	–	0.028	–
Total	1000.028	3798	1000.028	5358

Source: Adapted from Cintra et al. [145] e Razolli et al. [146]

Oxidative damage parameters in the hypothalamus

Oxidative damage was measured by the assessment of lipid peroxidation through the levels of malondialdehyde (MDA) and protein carbonylation in the hypothalamus. The formation of malondialdehyde, measured by high-performance liquid chromatography (Prominence Shimadzu on Ascentis® C18, 250 × 2.1 mm, 5 μm, Supelco Sigma-Aldrich column), was taken as a lipid peroxidation index, as described by Grotto et al. (2007). The results were expressed as MDA levels per milligram protein (nmol MDA/mg protein). The protein oxidation was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazone, as previously described (Levine et al. 1990). Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and dissolved in dinitrophenylhidrazone, and the absorbance was read at 370 nm. The results were expressed as protein carbonylation nmol/mg protein.

Antioxidant defense parameters in the hypothalamus

Antioxidant defense was measured by evaluating the activity of the superoxide dismutase (SOD) and catalase (CAT) enzymes, and by the glutathione levels in the hypothalamus. SOD (EC 1.15.1.1) activity was determined using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 32 °C (Bannister and Calabrese 1987). Absorption was measured at 480 nm. SOD specific activity was represented as mU/mg protein. CAT (EC 1.11.1.6) activity was assayed by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H2O2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 U/mg of protein (Aebi 1984). The specific activity was expressed as mU/mg protein. Glutathione (GSH) levels were determined using a technique described by Hissin and Hilf (1976). The technique is based on the color development resulting from the reaction between 5,5′-ditiobis-(2 nitrobenzoic acid) (DTNB) and thiols (absorbance reading at 412 nm). All results were expressed as units per milligram protein (U/mg protein).

Energy metabolism parameters

The energy metabolism was assessed by measuring the activity of complexes within the mitochondrial respiratory chain and the creatine kinase (CK) activity. Complex I activity was evaluated following the method described by Cassina and

Radi (1996) by measuring the rate of nicotinamide adenine dinucleotide (NADH)-dependent ferricyanide reduction. The reaction medium received the addition of potassium phosphate buffer, ferricyanide, NADH, rotenone and sample, and the reading was carried out in a spectrophotometer for 3 min at 420 nm. Complex II activity was measured by the method described by Fischer et al. (1985) by decreasing absorbance of 2,6-dicholorindophenol (2,6-DCIP). The sample was placed in an incubation medium containing potassium phosphate buffer, sodium succinate and DCIP. Incubation was carried out for 20 min at 30 °C in water bath. After incubation, sodium azide, rotenone and DCIP were added to samples, and then the spectrophotometric reading was performed for 5 min at 600 nm. Complex IV activity was determined according to the method described by Rustin et al. (1994) by measuring the decrease in absorbance caused by the oxidation of reduced cytochrome c. The incubation medium received the addition of potassium phosphate buffer, lauryl maltoside, sample diluted in sucrose, EDTA, trizme and heparin (SETH) buffer, and cytochrome c. After that, the reading was carried out in a spectrophotometer for 10 min at 550 nm. The colorimetric assay of CK activity was described by Hughes (1962). The reaction medium received the addition of diluted sample, lauryl maltoside, Tris buffer and phosphocreatine. After this, samples were placed in water bath at 37 °C. After 15 min adenosine diphosphate (ADP) was added, and samples were placed again in water bath for 10 min. Thereafter, phydroxymercuribenzoic (p-HMB) acid, alpha-naphthol, diacetyl and distilled water were added to the samples, which were placed in water bath again for 20 min. Finally, the creatine formed was measured using a spectrophotometer at 540 nm. All results were expressed as nanomoles of protein carbonylation per milligram of protein (nMol/mg protein).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism® 6.01 statistical software. Analyses were determined by the Student's t test, which compared the control group to the obese group. Statistical significance was set at $p < 0.05$.

Results

In the present study, obesity in male Swiss mice was induced by the consumption of a lipid-rich diet during a 10-week period. The animals in the obese group had a higher calorie intake than those of the control group (Fig. 1). Moreover, the obese group animals had higher body weight and higher mesenteric fat weight as compared to those of the control group (Fig. 1), thereby validating the animal model of obesity.

The hypothalamus was used for the evaluation of inflammatory parameters of oxidative stress and energy metabolism in mice. The animals in the obese group had an increase in IL-1β levels and a decrease in IL-10 levels in the hypothalamus, as compared to those of the control group (Fig. 2).

The results for the oxidative stress parameters, lipid peroxidation and protein carbonylation, showed that the animals in the obese group had higher oxidative damage in the hypothalamus than the control group (Fig. 3). Furthermore, the animals fed a high-fat diet showed a significant decrease in GSH levels in the hypothalamus. However, there was no statistically significant difference in the activity of antioxidant SOD and CAT enzymes (Fig. 3).

Finally, the effect of obesity on the energy metabolism in the hypothalamus of mice was evaluated. The animals fed a high-fat diet showed inhibition of complex I, II and IV activity of the mitochondrial respiratory chain in the hypothalamus. Moreover, the animals in the obese group had lower CK activity in the hypothalamus compared to the control group (Fig. 4).

Discussion

In order to investigate the influence of obesity on inflammatory and biochemical parameters in the hypothalamus of mice, the present study used an animal model of obesity based on the consumption of a high-fat diet. The consumption of saturated chain lipids is related to excess adiposity (Wang et al. 2012), changes in inflammatory markers, and dysregulation of metabolic enzymes. Based on these facts, many authors use high-fat diets to induce obesity in rodents (Kennedy et al. 2009).

Animals in the high-fat diet group consumed more calories than those on the normal-lipid diet. After 3 weeks consuming high-fat diet, the animals had gain more body weight than control group. The statistical difference was maintained until the end of the experiment. The obese mice also had a greater weight of mesenteric fat than control group. A similar profile has been described by Ma et al. (2014) in a study of saturated fat intake (40%) during 10 weeks, causing rapid weight gain and epididymal, perirenal and omental fat accumulation in rats.

With regard to inflammation and biochemical parameters, our data indicated that obesity in Swiss mice led to inflammation, oxidative damage and mitochondrial dysfunction in the hypothalamus. The obese animals showed lower levels of IL-10 in the hypothalamus as compared to animals fed with normal-lipid diet. This finding is consistent with Wang et al. (2012) who also found a decrease in IL-10 expression in the hypothalamus of obese animals (50% dietary saturated fat intake). Additionally, Van de Sande-Lee et al. (2011) have shown a significant increase in IL-10 levels in the cerebrospinal fluid of obese subjects after weight loss due to bariatric surgery. They also reported that IL-10 increasing was

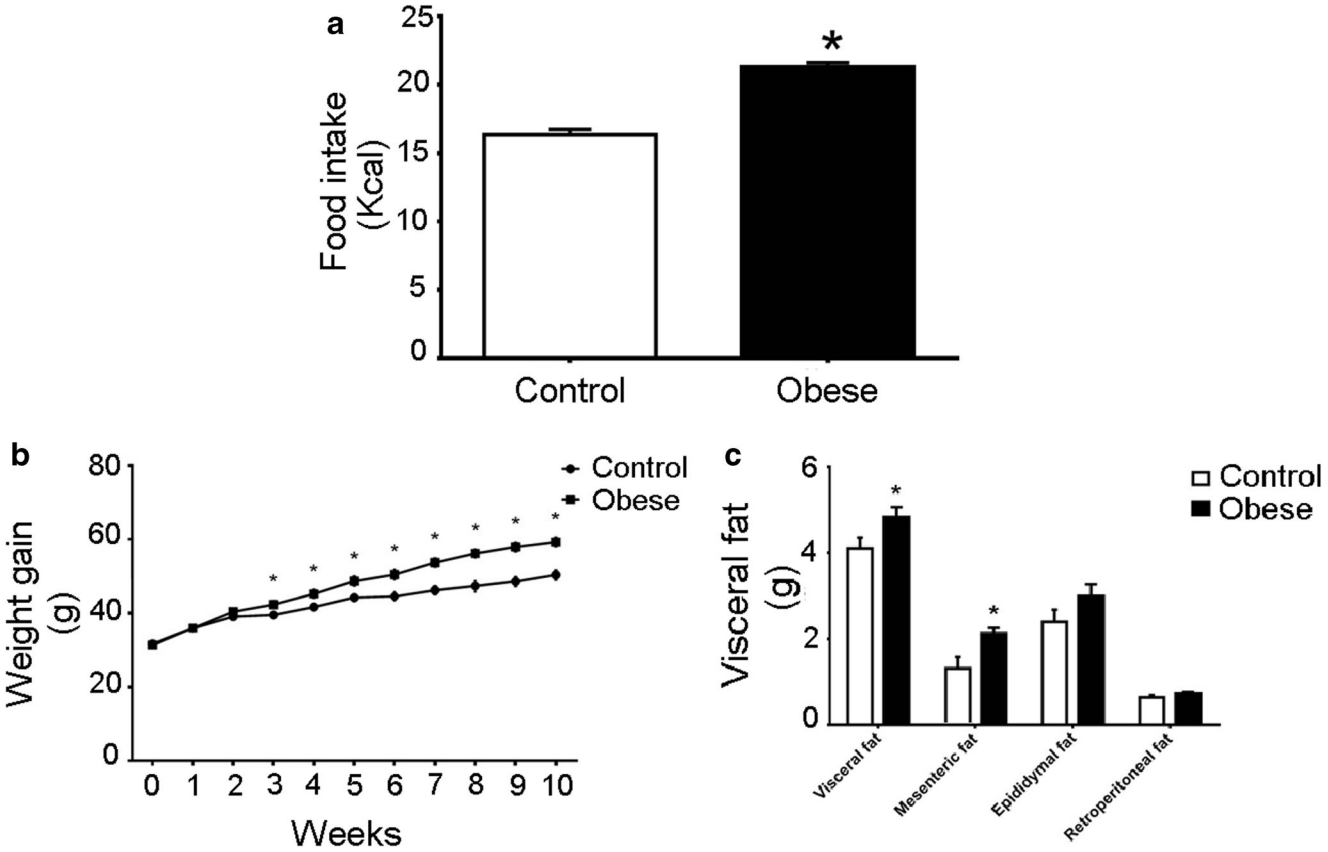

Fig. 1 Assessment of daily intake of calories (kcal) obtained by the consumption of a normal-lipid diet and high-fat diet (**a**) and effects of the intake of high-fat diet on body weight gain (**b**), as well as visceral fat mass in mice (**c**). Data were expressed as mean ± standard error of the mean ($n = 15$). *Different from the control group; $p < 0.05$

accompanied by changes in magnetic resonance patterns, especially in the hypothalamus.

In this study, the animals in the obese group showed higher IL-1β expression in the hypothalamus as compared to the control group. IL-1β is a cytokine that mediates the upregulation of many inflammatory cytokines, such as TNFα, by signaling through the IL-1β receptor, activating nuclear factor-kappa B (NF-kB) (Moynagh 2005). Several studies have demonstrated that animals fed with high-fat diet had increased expression of proinflammatory cytokines (including IL-1β), other inflammatory proteins, markers of glial activation, and markers of apoptosis in the hypothalamus (De Souza et al. 2005). Despite the methodological differences, this studies lead to the same conclusion: hypothalamic inflammation contributes to obesity pathogenesis (Kälin et al. 2015; Posey et al. 2009).

Evidence available to date does not clearly show when the inflammatory parameters are altered in the hypothalamus. It is

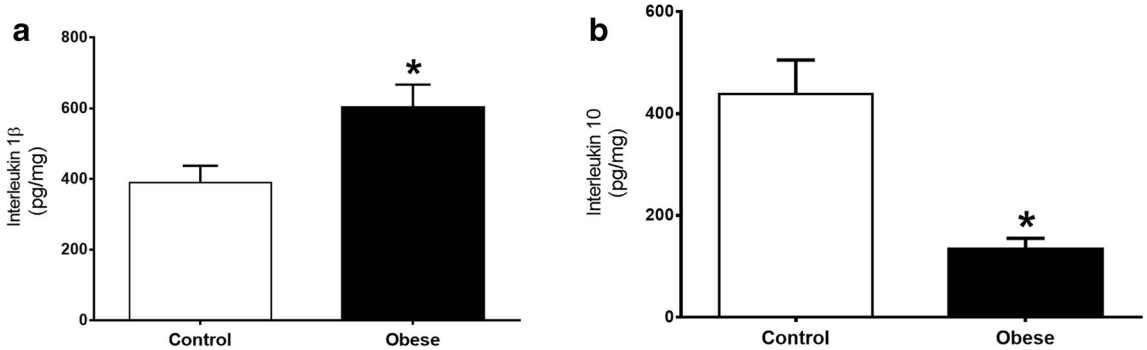

Fig. 2 Effect of obesity on the expression of IL-1β cytokine (**a**) and anti-inflammatory IL-10 cytokine (**b**) in the hypothalamus of mice. Data were expressed as mean ± standard error of the mean ($n = 5$). *Different from the control group; $p < 0.05$

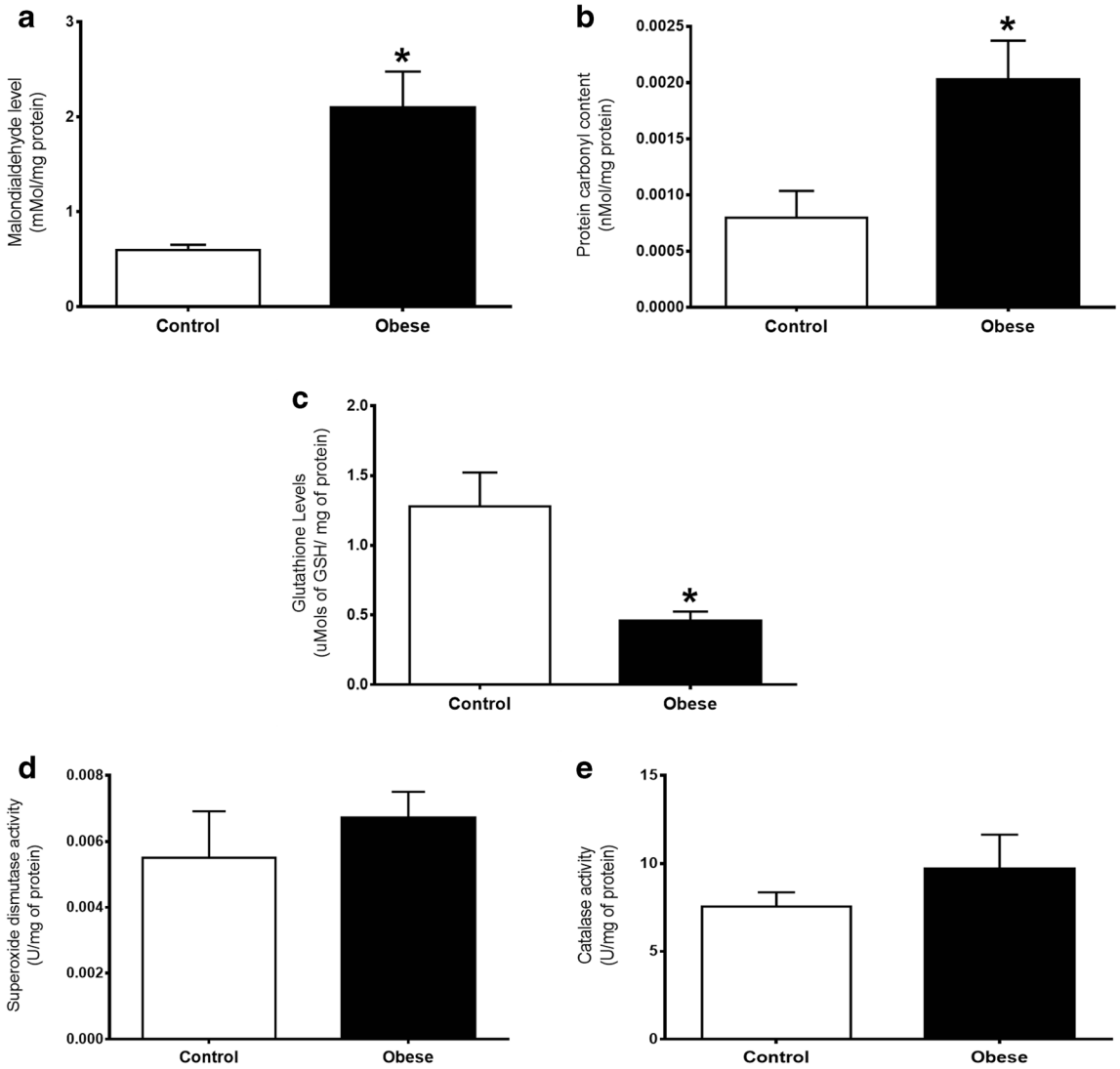

Fig. 3 Effect of obesity on oxidative stress markers in the hypothalamus of mice: Lipid peroxidation - MDA (**a**), protein carbonylation (**b**), GSH levels (**c**), superoxide dismutase activity (**d**), and catalase activity (**e**). Data were expressed as mean ± standard error of the mean ($n = 5$). *Different from the control group; $p < 0.05$

not clear whether systemic inflammation promotes inflammation in the hypothalamus or an initial hypothalamic damage would lead to brain impairment of food intake control with consequent fat accumulation. Thaler et al. (2012) have shown that animals fed a high-fat diet (60% dietary saturated fat intake) showed no increase in gene expression of inflammatory cytokines in the liver and adipose tissue before 4 weeks of diet-induced obesity. Contrastingly, inflammation in the hypothalamus was clearly evident after 3 days of a high-fat diet intake. They claimed that a high-fat diet affects the hypothalamus first, and then leads to systemic inflammation, which can take weeks (Thaler et al. 2013). In this study, we stated that obesity was associated with hypothalamic inflammation; however, we could not determine when this dysregulation began.

A high-fat diet compromises blood-brain barrier (BBB) integrity, thus facilitating the entry of immune cells in the CNS (Milanski et al. 2009). In this sense, high-fat diet and peripheral changes due body fat accumulation are associated with microglial activation and cytokine gene expression in CNS (Kälin et al. 2015). In a review article (Boitard et al. 2014), the authors have pointed out that NF-kB activation mediated by TNFα, in glial cells, was a key element for the harmful effects of this cytokine. All these mechanisms, including immune cell infiltration, as well as microglial activation, contribute to ROS production, with consequent oxidative damage (Kälin et al. 2015).

The data from this study indicated that the animals of the obese group had higher oxidative damage, in proteins and lipids, in the hypothalamus compared to the control group. Some aspects contribute to brain vulnerability to oxidative damage, as follows: (1) high metabolic rate and oxygen consumption; (2) high levels of polyunsaturated fatty acids, which

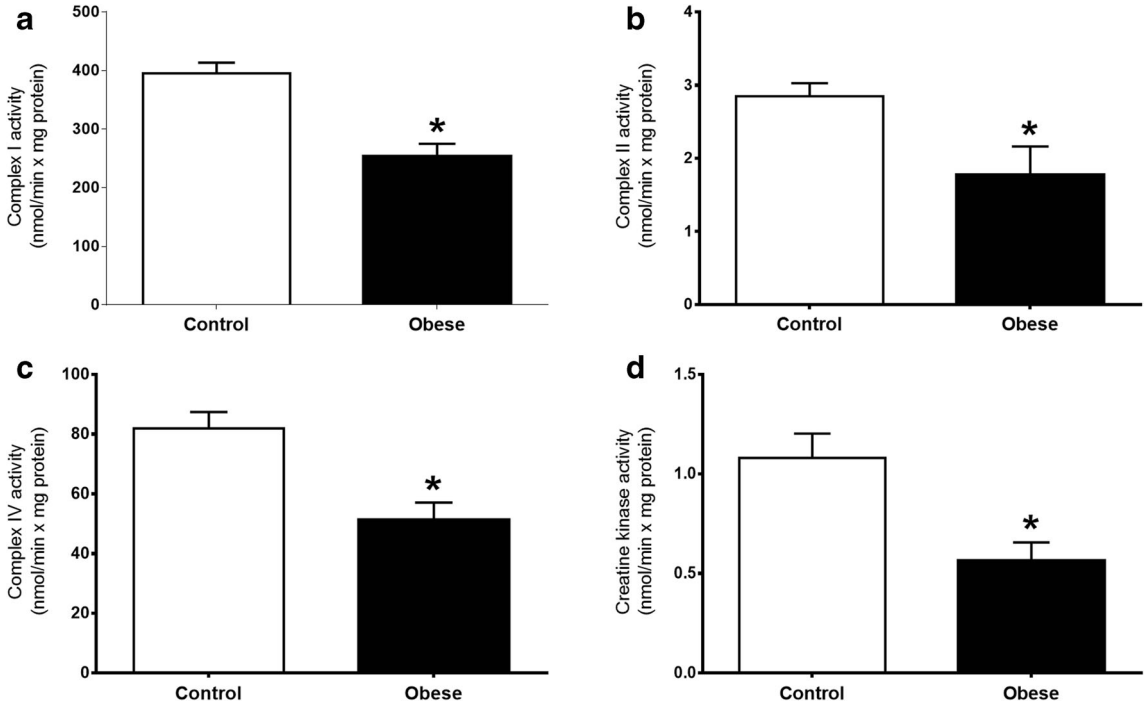

Fig. 4 Effects of obesity on the activity of Complex I (**a**), Complex II (**b**) and Complex IV (**c**) of the mitochondrial respiratory chain, and CK activity (**d**) in the hypothalamus of mice. Data were expressed as mean ± standard error of the mean (n = 5). *Different from the control group; $p < 0.05$

may be easily oxidated; and (3) high content of metals in the CNS, allowing ROS generation through Fenton's reaction (Barbosa et al. 2010).

The increase in oxidative agents in the brain is a great concern, given that chronic exposure to ROS can cause great damage to cell membranes, leading to loss of cell integrity and viability (Fischer and Maier, 2015; Lassmann 2011). On one hand, there are few studies showing the effect of saturated fat consumption in the hypothalamus; on the other hand, several studies have linked the consumption of a high-fat diet with hippocampus damage, associated with memory deficits and cognitive impairment (Boitard et al. 2014; Shefer et al. 2013; Sun et al. 2010).

In contrast to the large production of ROS, the CNS has low levels of antioxidant enzymes compared to other organs (Parkhurst and Gan 2010). In this study, obesity caused lower GSH levels in the hypothalamus. However, no alteration of SOD and CAT antioxidant activities. Freeman et al. (2013) have also assessed the influence of high-fat diet consumption (60% kcals from fat) on the antioxidant defense in the brain and found that there was no change in the activity of SOD and CAT enzymes in the hippocampus and prefrontal cortex, but glutathione peroxidase (GPx) activity showed a decline in the cerebral cortex.

GSH is used as a substrate for GPx to eliminate H_2O_2 and act in the detoxification of reactive aldehydes (such as MDA) that are generated during lipid peroxidation (Ward and Peters 1995). Therefore, the maintenance of GPx activity and adequate levels of GSH are of fundamental importance to prevent oxidative damage (Ward and Peters 1995). GSH activity is even more relevant in the mitochondria, given the large amount of ROS produced in that site. In this regard, evidence has shown that the depletion of mitochondrial GSH contributes to susceptibility to oxidative stress (Ward and Peters 1995). Ward and Peters (1995) have shown that in cerebellar neurons, depletion of mitochondrial and cytoplasmic GSH resulted in increased generation of ROS and mitochondrial function impairment. This information is consistent with our results, given that obese animals showed depletion in GSH levels along with reduced activity of complex I, II and IV of the mitochondrial respiratory chain.

It is well described in the literature that mitochondria play a central role in energy metabolism, and its main function and ultimately convert diet macronutrients into ATP (Heales et al. 1999). In this sense, the proper mitochondrial function is vital to cells (Aw and Jones 1989). Despite this great importance of energy metabolism in the cells, few studies have investigated this issue in the hypothalamus, and lead to loss of central control of food intake and the accumulation of body fat. It has already been demonstrated that high-fat diets are related to mitochondrial dysfunction in hypothalamic neurons, leading to decreased sensitivity to glucose levels (Wullner et al. 1999). Furthermore, findings from this study revealed a decrease in CK activity in the obese group, which indicates that

these animals had decreased ATP production in the hypothalamus. Colombani et al. (2009) demonstrated that obese rats had impaired hypothalamic regulation of glucose sensing, which is linked to an abnormal redox signaling, due mitochondrial dysfunction.

Taken together, all these data indicate that inflammation, oxidative stress and mitochondrial dysfunction are closely related to inappropriate consumption of saturated fat. The interrelationship between these processes comprises a complex feedback mechanism, which may cause cell damage in the hypothalamus, leading to lose control of food intake and accumulate body fat (De Souza et al. 2005).

The development of obesity is multifactorial (WHO 2015), not depending solely on the imbalance between caloric intake and energy expenditure. Thus, our data shows that the hypothalamus plays a central role in the development and progression of obesity.

Conclusion

From the results of this study, we can conclude that obesity is associated with the presence of inflammation, oxidative stress, and mitochondrial dysfunction in the hypothalamus of mice, since there was an increase in the levels of ROS and its components due to obesity. The interrelationship between these neuroinflammatory and neurochemical factors may have damaged hypothalamic cells, leading to loss of control over food intake and contributing to a continuous accumulation of body fat.

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