Deleterious effects of lard-enriched diet on tissues fatty acids composition and hypothalamic insulin actions

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Abstract

Background: high saturated fat intake is positively correlated with mild chronic inflammatory conditions such as type II diabetes, obesity and cardiovascular disease. Hypothalamic insulin signalling is intimately involved in food intake regulation, energy balance and maintenance of body weight. Previous studies report impaired hypothalamic insulin actions in obesity and type II diabetes.

Objectives: to investigate the effects of a high saturated fat diet on insulin hypothalamic signalling and insulin-induced hypophagia. The fatty acid composition of the hypothalamus and peripheral tissues have also been investigated.

Methods: 2 months old male Wistar rats were fed ad libitum with a standard rodent chow (15% of energy derived from fat, C), or lard-enriched chow (45% of energy derived from fat, HF) for 8 weeks. Subsequently, the animals were randomly grouped into two sets. One set was sacrificed and the hypothalamus, liver, retroperitoneal, epididymal and mesenteric fat pads, as well as trunk blood for the collection of serum, were obtained for fatty acid (FA) analysis by gas chromatography. The second set, after fasting, received an intracerebroventricular injection of insulin; the animals were sacrificed and their hypothalamus analysed for insulin signalling pathways by Western Blotting.

Results: Daily energy intake was similar between groups, but body weight and fat pad weight at the end of the 8 weeks treatment were significantly higher in HF (p<0.001). HF rats showed increased total saturated FA (SFA) content in serum (p<0.001) and adipose tissue (p<0.001), and decreased total monounsaturated FA (MUFA) in the hypothalamus (p<0.05). Total n-3 and n-6 polyunsaturated FA (PUFA) content was significantly decreased in all HF tissues and serum (p<0.01), but remained unchanged in the hypothalamus, which presented higher c20:5n-3 and c20:3n-6 (p<0.01). HF rats showed hyperglycaemia (p<0.05), impaired ability to inhibit food intake after intracerebroventricularly injected insulin (p<0.05), reduced IR tyrosine (p<0.05) and unchanged Akt serine phosphorylation (p<0.01) after insulin stimulation.

Conclusion: this study shows male rats treated for 8 weeks with a lard-enriched diet showed dramatic perturbations in peripheral tissue fatty acid composition. In HF hypothalamus, we found no changes in total SFA, increased total MUFA, no changes in total PUFA but increased C20:5n-3 and C20:3n-6, the respective C22:6n-3 and C20:4n-6 precursors. Impaired insulin actions on hypothalamic signalling pathways have been observed. It can be suggested peripheral tissues may have buffered the hypothalamic polyunsaturated fatty acid composition for the duration of this study, but not to the extent required for insulin action protection.
Introduction

The hypothalamus is one of the major structures of the central nervous system involved in metabolic sensing and hormone feedback. Appetite and energy expenditure are finely tuned by a combination of central and peripheral signals, including acute hormonal signals of hunger and satiety, as well as hormonal signals related to long-term control of adiposity (1, 2). Neural and hormonal inputs act in anabolic and catabolic hypothalamic effector systems, modulating food intake and energy expenditure in response to physiological needs in energy metabolism.

In the hypothalamus, insulin binds to insulin receptors present in areas involved in the control of food intake, including the arcuate nucleus and the ventromedial hypothalamus (3, 4, 5). The initial steps of insulin signalling in hypothalamic nuclei are similar to those in muscle and adipose tissues (6, 7). The insulin receptor (IR) belongs to a family of growth factor receptors, with two transmembrane subunits expressing intrinsic tyrosine kinase activity (8). Circulating insulin binds to the extracellular IR subunits, triggering its autophosphorylation in tyrosine residues, and stimulating the enzymatic activity of transmembrane subunits (9). Phosphorylated IR triggers a cascade of intracellular substrate phosphorylation, such as IR substrates (IRS) 1 and 2 (10).

Phosphorylated IRSs bind to and activate phosphatidylinositol-3-kinase (PI3-K), which in turn phosphorylates membrane phospholipids. Such actions are followed by activation of phosphatidylinositol-dependent protein kinases 1 and 2, and phosphorylation of protein kinase B (Akt) serine residues (8). Activation of the PI3-K pathway in hypothalamic nuclei mediates insulin-induced hypophagia, stimulating POMC neurons and inhibiting AgRP neurons, among other actions (11, 12).

Obesity has been associated with hypothalamic insulin resistance and hyperphagia (6, 13). Previous research showed consumption of a lard-enriched diet for 16 weeks increased IR serine phosphorylation, followed by higher expression of proinflammatory proteins in the hypothalamus of rats (14). Another study showed impaired hypothalamic Akt activation in rats treated with a butter-oil rich diet (15). Nevertheless, changes induced by high saturated fat diets on hypothalamic fatty acid composition, and their effects on hypothalamic insulin signalling, are yet to be fully understood.

A number of studies have demonstrated the deleterious effects of saturated fat rich diets on adipose tissue fatty acid composition and endocrine function (16-18). As cell membrane phospholipids are in intimate and functional contact with executive proteins, the fatty acid composition is decisive for tissue
stability and function (19, 20). Perturbations in tissue fatty acid composition have been reported in a number of health conditions, such as cardiovascular and metabolic diseases, cancer, neurodegenerative and behavioural disorders (21-24). These perturbations are manifested mainly by increased saturated, trans and omega 6 fatty acids, and concomitant decreased omega 3 fatty acids (5, 25-27).

In this study, we investigated in male rats the effects of a lard-enriched diet for 8 weeks in hypothalamus and peripheral tissue fatty acid composition. Hypothalamic insulin-related signalling pathways and insulin anorexigenic effects have also been investigated.

Methods

Animals

This study was approved by the Sao Paulo Federal University Research Ethics Committee, and all the procedures were carried out in full compliance with its ethical guidelines. Fifty eight male Wistar rats, weaned on the 21st day of life, were obtained from CEDEME (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia), and housed (four to five rats per cage) in the animal house of the Division of Nutrition Physiology, Sao Paulo Federal University, under controlled lighting (12 hours light/dark cycle, lights on at 6:00) and temperature (22 ± 1°C), with free access to standard rat chow and water. After two weeks acclimatization the rats were randomised into two groups, and fed a standard rat chow (C) or lard-enriched diet (HF) ad libitum for 8 weeks, as described below.

Diets

The diets were prepared in the food laboratory of the Division of Nutrition Physiology, as described previously (28). The standard rat chow used in this study (Nuvilab CR1, Nuvital, Brazil) was ground and enriched with lard, casein, sucrose and soybean oil, in the proportions 50/18/20/10/2. Casein was added to obtain a protein/energy ratio similar to the control diet, and soybean oil as a source of essential fatty acids. Lukewarm water was added to achieve the consistency necessary for perfect homogenization of the mixture, subsequently passed through a milling machine for the production of pellets, dried in a forced ventilation oven at 60°C for 24h. Samples of each diet were analysed in the laboratory of
Bromatology and Microbiology of Foods, Sao Paulo Federal University, for determination of macronutrients, and the diet fatty acid composition was determined by gas chromatography (Table 1).

Food and energy intake
Twenty-four hours food intake and body weight were measured weekly. Energy intake was calculated based on weekly food intake (g/100 g BW) and diet energy density (Kcal/g).

Tissue weight, glucose and insulin blood levels
At the end of the 8 weeks dietary intervention period, a set of rats from each group was sacrificed by decapitation without sedation after an overnight fast. Trunk blood was collected, serum immediately obtained by centrifugation and stored at -80°C until analysis. Retroperitoneal (RET), epididymal (EPI) and mesenteric (MES) fat pads, hypothalamus and liver were quickly dissected, weighted and immediately stored at -80°C. Serum glucose was determined by enzymatic colorimetric method (Glicose Pap Liquiform, Labtest Diagnóstica, Brazil) and insulin by radioimmunoassay (Rat insulin RIA kit, Millipore, USA).

Diet and tissue fatty acid composition
Total lipids were extracted from the control and lard-enriched diets, serum, hypothalamus, liver, RET, EPI and MES. Samples were homogenized with hexane/isopropanol (3:2 v/v) containing 0.01% butylated hydroxytoluene (BHT, Sigma, St Louis, MO), and washed with chloroform/methanol/water (2:1:1 v/v/v). After low speed centrifugation, the organic layer was obtained, evaporated to dryness under a stream of oxygen-free nitrogen (OFN), and lipids partitioned again in the chloroform layer of a chloroform/methanol/water solution (8/4/3 v/v/v). The resulting total lipid extracts were dried under OFN, sealed in air-tight tubes and dispatched in dry ice to the London Metropolitan University for fatty acid analysis.

Fatty acid analysis was performed as standardized previously (29). Briefly, fatty acid methyl esters (FAME) were obtained by heating the samples with 15% acetyl chloride in dry methanol in a sealed tube at 70°C for 3 hours under OFN. The reaction was stopped with the addition of 5% NaCl solution at room temperature, and FAMEs were extracted by washing three times with petroleum ether containing 0.01%
BHT. FAMEs were separated by gas chromatography (HRGC MEGA 2 series, Fisons Instruments, Milan, Italy) fitted with a capillary column (BPX70, Thames Restek, UK). Peak areas were quantified (EZChrom Chromatography Data System, Scientific Software Inc., San Ramon, CA, USA), and the values presented represent the percentage of total fatty acids.

**Intracerebroventricular insulin injection and food intake measurement**

The second set of C and HF rats were anesthetized with ketamine/xylazine (6.7/1.3 mg / 100g BW intraperitoneally), and stereotaxically implanted with a 21-gauge guide cannula aimed at the left lateral ventricle (from bregma: -0.9 mm anterior, +1.5 mm lateral, and -3.0 mm ventral) (30), as standardized in our laboratory (31). Animals were individually caged thereafter with free access to their allocated chow and water. Four days after surgery, correct cannula placement was confirmed by a positive drinking response after a 20 ng angiotensin II intracerebroventricular injection. One week after surgery, C and HF rats received a 2 µL intracerebroventricular injection of 20 mU insulin (Humulin, Eli Lilly, USA) or saline after 6 hours fasting. Injections were performed immediately before lights went off, and a known amount of their respective chow was offered. The chow consumed was quantified after 12 and 24 hours.

**Evaluation of hypothalamic insulin signalling pathway by Western Blotting**

One week after the experiment described above took place, rats were deprived of food for 6 hours, lightly anesthetized with ketamine/xylazine intraperitoneally, and intracerebroventricularly injected with 200 mU of insulin (Humulin, Eli Lilly, USA) or saline. Fifteen minutes later, rats were decapitated, the hypothalamus quickly removed and immediately homogenized in 1.0 mL of solubilisation buffer (10 mM EDTA, 100 mM Tris pH 7.5, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 2 mM PMSF, 2 μg/mL aprotinin, 1% Triton X-100). Insoluble material was removed by centrifugation (40 min, 14000 rpm, 4°C). Supernatant protein concentration was determined colorimetrically (BCA Protein Assay, Bioagency Biotecnologia, Brazil). Tissue extracts (100 µg) were denatured in boiling water for 5 minutes in Laemmli buffer (32) containing 200 mM DTT.

Protein extracts were separated by SDS-PAGE, transferred onto nitrocellulose membrane and incubated with primary antibody anti IR-β (sc-711), p-IR-β (sc-25103), IRS-1 (sc-559) (Santa Cruz Biotechnologies, USA), IRS-2 (3089), Akt (9272), p-Akt (9271) (Cell Signaling, USA). For evaluation of protein loading, all membranes were stripped and rebotted with anti-α-tubulin primary antibody (sc-58667). After
Statistical analysis

Results are expressed as mean and standard error. Comparisons between groups (C vs. HF) were assessed using independent samples Student’s t tests, equal variances assumed. Phosphorylation data differences were assessed by one-way ANOVA followed by Tukey’s HSD (Honestly Significant Difference) post hoc tests for multiple comparisons. Statistical significance was set at p<0.05.

Results

Food and energy intake and body weight

60-day old male Wistar rats were treated for 8 weeks with a standard rat chow (C) or a lard-enriched diet (HF). During the course of the 8 weeks treatment, weekly-measured 24h food intake (g/100g BW) was significantly lower in HF (p<0.05 week 1, p<0.01 week 2 and thereafter), energy intake was statistically similar between groups in week 2 and thereafter, and body weight was significantly higher in HF (p<0.05 week 2 and 3, p<0.01 week 4 and thereafter) (Figure 1).

Fat pad mass and serum glucose and insulin

The HF group showed highly significant increases in RET, EPI and MES masses (p<0.001) (table 1), higher blood glucose (p<0.05) and similar insulin (table 2).

Fatty acids profile of diets and tissues

Diets

The HF diet contained more than double the amount of total SFA (32.8%) as compared to the C diet (14.8%), and 1.5 times higher amount of total MUFA (40.5%) as compared to C (25.9%). The HF diet contained only 33% of the c18:3n-3 (C 4.1%; HF 1.4%) and 42% of the c18:2n-6 (C 54.9%; HF 23.1%) content as compared to C. The ratio n-6/n-3 is approximately 27% higher in HF (C 13.3; HF 16.6) (table 1).
**Hypothalamus**

After 8 weeks of lard-enriched diet consumption, the HF hypothalamus showed decreased amount of total MUFAs (p<0.05) but increased ratio of C18:0/C18:1 (p<0.05), an indirect index of enhanced activity of the microsomal enzyme stearyl-CoA desaturase (delta 9 desaturase). Higher levels of C20:3n-6 (p<0.01) and lower C22:5n-6 (p<0.001) were also found in HF rats (table 3). Higher levels of C20:5n-3 (p=0.015), and a tendency to increased C22:6n-3 (p=0.08) were found in HF rats.

**Serum**

Lower C14:0 (p<0.01) and C16:0 (p<0.05) were found in the HF group, followed by substantially higher C18:0 (p<0.001) and total SFA (p<0.001). The ratio C16:0/C18:0 is significantly decreased in HF (p<0.001), which could be attributed to a reflection of dietary provision or increased elongase activity. The HF treatment decreased C16:1n-7 (p<0.05) and C18:1n-7 (p<0.001), but did not change other MUFAs nor the total MUFA content; however, it increased the ratio C18:0/C18:1 (p<0.01). Lower amounts of C18:3n-3 (p<0.001), C20:5n-3 (p<0.01), C22:5n-3 (p<0.01) and total n-3 PUFA (p<0.01) were found in the HF group. The HF treatment lowered the amounts of C18:2n-6 (p<0.001) and C20:3n-6 (p<0.05), but increased C20:4n-6 (p<0.01) and C22:5n-6 (p<0.001); the total n-6 PUFA remained significantly decreased (p<0.01). The total PUFA content (n-6 + n-3) was decreased (p<0.01), the ratio n-6/n-3 was increased (p<0.01) and the ratio SFA/PUFA was increased (p<0.001).

**Liver**

A significant decrease in C24:0 (p<0.01) was found in the HF group, but this fatty acids accounts for less than 0.5% of total fatty acids; no significant differences were found in other SFAs. Higher C18:1n-9 (p<0.01), C20:1 (p<0.05) and total MUFA (p<0.01) were found in the HF group, followed by lower C18:1n-7 (p<0.001). As opposed to serum and hypothalamus, the ratio C18:0/C18:1 was not significantly higher in HF. All the n-3 fatty acids quantified were significantly reduced (p<0.05) in the liver of HF rats, but only C20:5n-3 did not reach statistical significance (p=0.39) despite an approximate 30% decreased amount. The HF treatment induced a more complex variation in the n-6 fatty acid amounts: C18:2n-6 (p<0.05), C20:4n-6 (p<0.05) and the total n-6 PUFA (p<0.01) were lower, but C18:3n-6 (p<0.01), C22:4n-6 (p<0.01) and C22:5n-6 (p<0.01) were higher. C20:3n-6 (the C20:4n-6 precursor) was similar between HF and C.
The total PUFA content (n-6 + n-3) was lower (p<0.01), the ratio n-6/n-3 (p<0.05) and SFA/PUFA (p<0.001) were higher in HF rats.

**Retroperitoneal, epididymal and mesenteric fat pads**

The three white fat pads analysed were dramatically affected by the HF diet, and showed very similar patterns of variation. C16:0 (p<0.05 at least), C18:0 (p<0.001) and total SFA (p<0.001) were higher in HF, and the ratio C16:0/C18:0 was lower (p<0.001) in all three white fat pads. C16:1n-7 was lower (p<0.05) in HF RET only, but C18:1n-9 (p<0.001), C20:1 (p<0.001), total MUFA (p<0.001) and the ratio C18:0/C18:1 (p<0.01 at least) were higher in all three HF fat pads. C18:3n-3, C22:5n-3, C22:6n-3 and total n-3 PUFA were lower (p<0.001) in all HF fat pads. No significant differences were found in C20:5n-3, despite an approximate 33% reduction in EPI, 17% in RET and 40% in MES. C18:2n-6 (p<0.001), C18:3n-6 (p<0.001), C20:3n-6 (p<0.01 at least), C20:4n-6 (p<0.001) and C22:4n-6 (p<0.01 at least) were lower in HF fat pads; C22:5n-6 was lower only in EPI (p<0.05). The total n-6 PUFA (p<0.001), total PUFA (p<0.001), ratio n-6/n-3 (p<0.001) and ratio SFA/PUFA (p<0.001) were lower in the HF group.

**Insulin-induced hypophagia**

C showed significantly reduced food intake (p<0.05) on the first 12 hours after intracerebroventriculally injected insulin, as compared to saline injection. However, insulin injection showed no effect in the HF group. On the second 12 hours period, the appetite-suppressing effect of insulin had subsided in C, and no retarded effect was observed in HF. The cumulative 24h intake was significantly decreased (p<0.05) after insulin injection in C, an overall 19% reduction, but no significant effect was found in HF (Figure 2).

**Hypothalamic insulin signalling pathway**

IR protein levels were significantly reduced (p<0.05) in HF (Fig 3A). IR tyrosine phosphorylation (pIR) was significantly increased after insulin injection in C (p<0.05), a 143% increase; however, this effect was not observed in HF, who showed a statistically insignificant, 14% increase (Fig 3B). IRS-1 and IRS-2 protein levels were similar between groups (data not shown). Akt protein levels were significantly lower in HF (p<0.05) (Fig 3C). Akt serine phosphorylation was significantly increased after insulin injection in C (49%
increase, p<0.01) but the same effect was not observed in HF, who showed a statistically insignificant, 15% increase (Fig 3D).

**Discussion**

Obesity is a well characterized Public Health issue, and its correlation with major health issues is clear. Enlarged adipose mass leads to increased peripheral vascular resistance, macrophage infiltration, metabolic syndrome and mild chronic inflammatory conditions (33-35). Chronic positive energy balance is the main causal factor for the development of obesity, but often the abundant intake of affordable, palatable, energy-dense foods triggers a nutritional imbalance that may be correlated with metabolic disorders. In the present study, male Wistar rats treated for 8 weeks with a lard-enriched diet (52% energy derived from fat) showed increased body and adipose tissue masses, as compared to rats fed a standard chow (15% energy from fat). These results confirm previous studies describing the effects of high fat diets as inducers of obesity in animal models and humans (16, 36-38), and the obesogenic effects of saturated fat (39, 40). Several molecular mechanisms have been associated with increased adiposity induced by high fat diets, including reduced rate of dietary fat oxidation, higher lipogenesis and lower lipolysis rates, increased uptake of circulating lipids due to higher lipoprotein lipase activity (41-43), and others.

In this study, HF rats showed lower food intake, but similar energy intake, as compared to C, from the second week of treatment onwards. Previous studies have shown rats treated with high fat diets showed increased body fat content and decreased food intake (42, 44). It has also been shown the consumption of diets rich in saturated fat reduced the expression of the orexigenic peptides neuropeptide Y (45) and agouti-related protein (46) in the arcuate nucleus of hypothalamus, and increased the secretion of the anorexigenic cholecystokinin (47).

After 8 weeks of dietary treatment, HF rats showed increased fasting glycaemia alongside normoinsulinemia, which suggests the development of peripheral insulin resistance, in agreement with previous studies (40, 48). The type of fat appears to be decisive for the development of insulin resistance: mice treated with either MUFA or SFA rich diets showed increased body mass, but only those treated with SFA developed glucose intolerance (49). In corroboration with the latter, mice treated for 60 days with a lard-enriched diet showed decreased adiponectin serum levels (27), an adipokine directly related to insulin sensitivity and glucose homeostasis.
The HF chow used in this study provided more than double the amount of total SFA as compared to the C chow. The ratio n-6/n-3 was 17:1 in the HF diet, and 13:1 in the C diet, but the HF diet contained only 33% of the total n-3, and only 42% of the total n-6, present in the C diet. As expected, tissue fatty acid composition has been dramatically changed in HF rats. Previous studies have shown the influence of dietary fats in the fatty acid composition of several tissues, including skeletal muscle, subcutaneous fat, liver, heart, pancreas, stomach, intestine and plasma (50), as well as on the development of metabolic and brain disorders (22, 51).

The fatty acid composition of the HF white fat pads analysed in this study showed dramatic variation in relation to C: the amounts of n-6 PUFAs were significantly decreased, replaced by substantially higher amounts of SFAs and MUFAs. The SFA geometrical configuration increases membrane phospholipid rigidity and lipid bilayer tension, which impairs GLUT4 vesicle exteriorization, resulting in glucose intolerance (21, 52). Diabetes-induced glucotoxicity contributes to β-cells failure, and exposing cultured human pancreatic islets to MUFAs reduced apoptosis induced by high glucose and palmitic acid levels (53). Additionally, a recent study showed that a butter-enriched diet had a detrimental impact on the indirectly measured activity of stearoyl-Coa desaturase 1, which correlated with metabolic disorders and obesity (54).

In the present study, all HF peripheral tissues and serum showed dramatically decreased n-3 PUFA levels; however, such changes were not reflected in the hypothalamus, which on the contrary showed significantly higher levels of C20:5n-3 and C20:3n-6 (p<0.01). These findings support the hypothesis peripheral tissues store essential fatty acids over periods or dietary abundance, supplying them to central tissues in periods of dietary deficiency.

In addition to the above, the hypothalamus showed a small but significant decrease in total MUFA, despite higher MUFA levels in the diet. Previous studies have found an inverse correlation between cerebral spinal fluid and whole blood C18:1n-9 in humans (55, 56). Interestingly, a literature review clarified shifting a SFA-rich diet to a MUFA-rich diet improved insulin sensitivity, whereas a moderate supplementation with n-3 PUFA did not have the same level of significance on insulin actions (20). In a rodent model of obesity, saturated fat feeding induced hypothalamic cell apoptosis by activation of Toll-like receptor 4, a member of the IL-1/Toll receptor family playing important roles in host defence and inflammatory processes (57).

Impaired insulin-mediated signalling actions in neural systems directly impacts on the development of metabolic diseases. The anorexigenic effects of insulin are decisive for the maintenance of body energy
homeostasis, and deficient insulin signalling has been associated with obesity in various experimental models (6, 48, 58). In the present study, we examined whether a 8 weeks lard-enriched dietary intervention affected food intake after insulin intracerebroventricular administration. Insulin significantly reduced food intake in C, but no actions observed in HF, in agreement with previous research (14, 15).

Additionally, we found decreased hypothalamic IR protein levels in HF rats. Other studies, employing different experimental models and looking into gene expression, have found either increased (48) or unchanged (14) IR levels. Previous research from our group showed unchanged IR protein levels in rats treated with soybean or fish oil enriched diets, as compared to standard chow fed rats. (4) Even though we did not investigate gene expression, but protein amounts instead, it is plausible to suggest that the lack of essential fatty acids impaired the synthesis, translocation and or exteriorization of IR protein.

In this study, HF rats did not show significantly increased IR tyrosine phosphorylation after stimulation with intracerebroventricularly injected insulin, as in C rats (Fig 3B). A previous study suggested increased IR serine phosphorylation may be an important factor in reducing IR tyrosine phosphorylation (6).

We did not find any differences in hypothalamic levels of IRS-1 and IRS-2 (data not shown). It has been shown obese, insulin-resistant Zucker rats presented moderately decreased IRS-1 and IRS-2 hypothalamic expression (6). On the other hand, it has also been shown that consumption of a saturated fat rich diet for 1 week increased IR, IRS-1 and 2 gene expression in rat hypothalamus, but such perturbations subsided after 6 weeks on that diet, suggesting the hypothesis of an adaptive mechanism (48).

Protein kinase B (Akt), the more distal insulin-signalling cascade protein investigated in this study, is regulated by several molecular mechanisms, and plays a key role in relevant biological processes such as apoptosis, cell proliferation and differentiation (59, 60), glucose uptake in peripheral tissues (61, 62), and activation of hypothalamic anorexigenic neurons (63, 64). In this study, we found lowered Akt protein levels in HF (Fig 3C), increased Akt serine phosphorylation in C after intracerebroventricularly injected insulin, and no effect in HF (Fig 3D). We therefore propose the imbalance in fatty acid tissue composition is responsible for such impairment in HF. In agreement with our proposition, previous studies have shown insulin is less effective in inducing satiety and Akt phosphorylation in the hypothalamus of rats fed with saturated fat-rich diets (15, 65). Hypothalamus inflammation induced by saturated fat-rich diet has been defined as a relevant factor for the impaired insulin signalling (66).

We found increased C20:5n-3 (the C22:6n-3 precursor) and C20:3n-6 (the C20:4n-6 precursor) in HF total hypothalamus lipid extract. Both C22:6n-3 and C20:4n-6 remained unchanged in HF, as well as the total
n-3 and total n-6 PUFAs. C22:6n-3 and C20:4n-6 are the main fatty acids in the medial basal hypothalamus of cows (67), and the main fatty acids in major neural systems in humans (68, 69). We hypothesise increased C20:5n-3 and C20:3n-6, followed by unchanged C22:6n-3 and C20:4n-6 in HF hypothalamus is the result of a protective mechanism in which peripheral tissues supply these fatty acids, as evidenced by their reduced levels in peripheral tissues. Although interesting, such mechanism may not have been efficient enough to prevent hypothalamic insulin-signalling impairment. Nonetheless, we have not investigated other molecular pathways in this study, and we do not know whether a HF treatment for longer than 8 weeks could induce changes in hypothalamic PUFA levels.

The results presented in this study support previous reports of central insulin resistance after dietary manipulations leading to increased body adiposity. Impaired insulin-induced hypophagia, as well as impaired hypothalamic insulin signalling, have been shown in adult rats submitted to intrauterine food restriction, in rats fed a trans fatty acid-enriched, and in rats fed a soybean oil-enriched diet (4, 25, 59).

In summary, despite showing similar energy intake, rats treated with a lard-enriched diet for 8 weeks showed higher body mass, adiposity and glycaemia as compared to control rats. All peripheral tissues analysed in this study showed dramatic perturbations in their fatty acid composition, showing significantly higher SFA and lower n-3 and n-6 PUFAs. However, the hypothalamus was not as much affected, showing levels of total PUFAs similar to C. HF rats did not show the same level of insulin-induced hypophagia as C, which is likely to be explained by impaired insulin signalling. HF rats also showed reduced levels of IR phosphorylation.

The chronic intake of a diet high in SFA and low in PUFA induced deleterious effects on peripheral metabolism and on insulin central actions. These results contribute to the elucidation of molecular mechanisms involved in appetite control, energy homeostasis and the development of major metabolic disorders such as obesity and type II diabetes.

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Authors’ contribution

APSD, RLHW and AAB designed the research, conducted laboratory work, analysed the results and wrote the paper. GDP, VTB and YW conducted laboratory work, analysed the results and wrote the paper. CMON and LMO designed the research, analysed the results and wrote the paper. EBR is the Principal Investigator, designed the research, analysed the results and wrote the paper. All authors commented on the final versions of the paper, and share primary responsibility for its final content.
**Figure legends**

**Figure 1:** Food (g / 100g bw / 24h, upper panel) and energy (Kcal / 100g bw / 24h, middle panel) intake and body weight (g, lower panel) of male rats treated with a standard rodent chow (2.7 Kcal/g, 15% energy from fat) or lard-enriched chow (4.1 Kcal/g, 52% energy from fat) for 8 weeks. Error bars indicate standard error of the mean. N = 20 for each group.

* p < 0.05 HF vs. control; ** p < 0.01 HF vs. control; *** p < 0.001 HF vs. control

**Figure 2:** Food intake during the first and second 12-hour periods, and during the entire 24 hours period after intracerebroventricular injection of vehicle (solid bars) or insulin (striped bars) of male rats treated with a standard rodent chow (2.7 Kcal/g, 15% energy from fat) or lard-enriched chow (4.1 Kcal/g, 52% energy from fat) for 8 weeks. Error bars indicate standard error of the mean. C, n = 23-28; HF, n=19-21.

# p < 0.05 vs. respective vehicle-treated group.

**Figure 3:** Top panels (A and B): hypothalamic insulin receptor (IR) protein levels (a) and tyrosine phosphorylation (b) of control (C, n=7-8) and high-fat (HF, n=8-9) groups. Bottom panels (C and D): hypothalamic Akt protein levels (a) and serine phosphorylation (b) of control (C, n=6-8) and high-fat (HF, n=6-7) groups. Rats were treated with intracerebroventricularly injected vehicle (-) or insulin (+). Error bars indicate standard error of the mean.

* p < 0.05 HF vs. control; # p <0.05 vs. the respective saline-treated group; # # p < 0.001 vs. the respective saline-treated group.
### Table 1: Total calorie value (Kcal/g), protein content, carbohydrates, alimentary fibre, mineral residues (g/100g) and fatty acid composition (% of total fatty acids) of control chow and lard-enriched chow.

<table>
<thead>
<tr>
<th></th>
<th>Control chow</th>
<th>Lard-enriched chow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (Kcal/g)</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Protein (g/100g)</td>
<td>22.4</td>
<td>23.6</td>
</tr>
<tr>
<td>Carbohydrates (g/100g)</td>
<td>39.1</td>
<td>26.8</td>
</tr>
<tr>
<td>Alimentary fibre (g/100g)</td>
<td>11.4</td>
<td>15.1</td>
</tr>
<tr>
<td>Mineral residues (g/100g)</td>
<td>11.9</td>
<td>9</td>
</tr>
<tr>
<td>Total fat</td>
<td>4.8</td>
<td>22</td>
</tr>
<tr>
<td>Fatty acid composition (% total)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c14:0</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>c16:0</td>
<td>12.3</td>
<td>20.9</td>
</tr>
<tr>
<td>c18:0</td>
<td>2.4</td>
<td>10.9</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>14.8</td>
<td>32.8</td>
</tr>
<tr>
<td>ratio c16:0/c18:0</td>
<td>5.1</td>
<td>1.9</td>
</tr>
<tr>
<td>c16:1n7</td>
<td>0.0</td>
<td>1.7</td>
</tr>
<tr>
<td>c18:1n9</td>
<td>24.6</td>
<td>36.0</td>
</tr>
<tr>
<td>c18:1n7</td>
<td>1.1</td>
<td>2.3</td>
</tr>
<tr>
<td>c20:1</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>ΣMUFA</td>
<td>25.9</td>
<td>40.5</td>
</tr>
<tr>
<td>c18:3n3</td>
<td>4.1</td>
<td>1.4</td>
</tr>
<tr>
<td>c18:2n6</td>
<td>54.9</td>
<td>23.1</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>59.0</td>
<td>24.5</td>
</tr>
<tr>
<td>Σn-6/Σn-3</td>
<td>13.3</td>
<td>16.6</td>
</tr>
<tr>
<td>ΣSFA/ΣPUFA</td>
<td>0.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Table 2: Fat pad weight (g / 100 g BW) and serum glucose (mg / dL) and insulin (ng / mL) of male rats treated with a standard rodent chow (C, 2.7 Kcal/g, 15% energy from fat) or lard-enriched chow (HF, 4.1 Kcal/g, 52% energy from fat) for 8 weeks. Data are presented as means ± SEM, n = 14 for C; n = 9-16 for HF.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fat pad weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>0.71±0.04</td>
<td>1.40±0.09***</td>
</tr>
<tr>
<td>Epididymal</td>
<td>0.82±0.05</td>
<td>1.47±0.10***</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>0.70±0.04</td>
<td>1.30±0.08***</td>
</tr>
<tr>
<td><strong>Serum glucose</strong></td>
<td>104.7±3.7</td>
<td>119.6±6.3*</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum insulin</strong></td>
<td>0.8±0.1</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05 HF vs. control; ** p < 0.01 HF vs. control; *** p < 0.001 HF vs. control
Table 3: Fatty acid composition of liver, serum and hypothalamus total lipid extract of male Wistar rats treated with a standard rodent chow (C, 2.7 Kcal/g, 15% energy from fat) or lard-enriched chow (HF, 4.1 Kcal/g, 52% energy from fat) for 8 weeks. Data are presented as means ± SEM of total FAs (%). n = 5 for each group.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Serum</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>SE</td>
<td>HF</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.3</td>
<td>±0.04</td>
<td>0.2</td>
</tr>
<tr>
<td>C16:0</td>
<td>17.6</td>
<td>±0.48</td>
<td>16.8</td>
</tr>
<tr>
<td>C18:0</td>
<td>14.7</td>
<td>±0.44</td>
<td>16.1</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.5</td>
<td>±0.02</td>
<td>0.3</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>33.0</td>
<td>±0.51</td>
<td>33.4</td>
</tr>
<tr>
<td>C16:0/C18:0</td>
<td>1.2</td>
<td>±0.06</td>
<td>1.1</td>
</tr>
<tr>
<td>C16:1n-7</td>
<td>0.7</td>
<td>±0.15</td>
<td>0.4</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>7.3</td>
<td>±0.25</td>
<td>17.5</td>
</tr>
<tr>
<td>C18:1n-7</td>
<td>3.3</td>
<td>±0.17</td>
<td>2.0</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.2</td>
<td>±0.02</td>
<td>0.3</td>
</tr>
<tr>
<td>C24:1</td>
<td>0.2</td>
<td>±0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>ΣMUFA</td>
<td>11.7</td>
<td>±0.43</td>
<td>20.3</td>
</tr>
<tr>
<td>C18:0/C18:1</td>
<td>1.4</td>
<td>±0.07</td>
<td>0.9</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.3</td>
<td>±0.03</td>
<td>0.2</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>0.3</td>
<td>±0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>C22:5n-3</td>
<td>1.5</td>
<td>±0.11</td>
<td>1.1</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>5.0</td>
<td>±0.49</td>
<td>3.7</td>
</tr>
<tr>
<td>Σn-3</td>
<td>7.1</td>
<td>±0.37</td>
<td>5.2</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>18.0</td>
<td>±0.74</td>
<td>15.5</td>
</tr>
<tr>
<td>C18:3n-6</td>
<td>0.3</td>
<td>±0.01</td>
<td>0.4</td>
</tr>
<tr>
<td>C20:3n-6</td>
<td>0.6</td>
<td>±0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>25.1</td>
<td>±0.39</td>
<td>20.1</td>
</tr>
<tr>
<td>C22:4n-6</td>
<td>0.9</td>
<td>±0.03</td>
<td>1.5</td>
</tr>
<tr>
<td>C22:5n-6</td>
<td>0.2</td>
<td>±0.05</td>
<td>0.9</td>
</tr>
<tr>
<td>Σn-6</td>
<td>45.0</td>
<td>±0.70</td>
<td>38.9</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>52.1</td>
<td>±0.75</td>
<td>44.1</td>
</tr>
<tr>
<td>Σn-6/Σn-3</td>
<td>6.4</td>
<td>±0.35</td>
<td>7.5</td>
</tr>
<tr>
<td>ΣSFA/ΣPUFA</td>
<td>0.6</td>
<td>±0.02</td>
<td>0.8</td>
</tr>
</tbody>
</table>

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ND: not detected. p < 0.05 *; p < 0.01 **; p < 0.001 ***.
Table 4: Fatty acid composition of retroperitoneal, epididymal and mesenteric white adipose tissue total lipid extract of male Wistar rats treated with a standard rodent chow (C, 2.7 Kcal/g, 15% energy from fat) or lard-enriched chow (HF, 4.1 Kcal/g, 52% energy from fat) for 8 weeks. Data are presented as means ± SEM of total FAs (%). n = 5 for each group.

<table>
<thead>
<tr>
<th>EPI</th>
<th>RET</th>
<th>MES</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>SE</td>
<td>HF</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.8</td>
<td>± 0.05</td>
</tr>
</tbody>
</table>
| C16:0| 20.3| ± 0.31| 22.0| ± 0.23| **| 20.8| ± 0.23| 21.8| ± 0.12| **| 19.3| ± 0.39| 20.2| ± 0.12| *
| C18:0|  2.7| ± 0.14|  5.9| ± 0.23| ***|  3.0| ± 0.04|  6.3| ± 0.05| ***|  3.2| ± 0.09|  7.8| ± 0.21| ***
| C24:0| ND  | ND  | ND | ND | ND  | ND  | ND  | ND | ND |
| ∑SFA | 23.9| ± 0.37| 28.8| ± 0.12| ***| 24.6| ± 0.21| 28.9| ± 0.13| ***| 23.2| ± 0.45| 28.8| ± 0.19| ***
| C16:0/C18:0| 7.5| ± 0.42| 3.7| ± 0.17| ***| 7.0| ± 0.14| 3.5| ± 0.04| ***| 6.0| ± 0.18| 2.6| ± 0.08| ***
| C16:1n-7| 2.8| ± 0.50| 2.4| ± 0.16|  2.1| ± 0.08|  1.7| ± 0.10|  *|  1.3| ± 0.12|  1.2| ± 0.02| *
| C18:1n-9| 24.2| ± 0.20| 39.8| ± 0.24| ***| 25.0| ± 0.30| 41.4| ± 0.30| ***| 25.9| ± 0.30| 42.2| ± 0.16| ***
| C18:1n-7|  2.7| ± 0.12|  2.8| ± 0.03| ***|  2.7| ± 0.15|  2.8| ± 0.05| ***|  2.7| ± 0.10|  2.9| ± 0.04| *
| C20:1|  0.2| ± 0.01|  0.3| ± 0.01| ***|  0.2| ± 0.01|  0.4| ± 0.02| ***|  0.2| ± 0.01|  0.5| ± 0.01| ***
| C24:1| ND  | ND  | ND | ND | ND  | ND  | ND  | ND | ND |
| ∑MUFA | 29.9| ± 0.68| 45.3| ± 0.22| ***| 30.0| ± 0.41| 46.4| ± 0.19| ***| 30.1| ± 0.45| 46.8| ± 0.17| ***
| C18:3n-3| 2.4| ± 0.06| 0.9| ± 0.01| ***|  2.3| ± 0.08|  0.7| ± 0.03| ***|  2.0| ± 0.11|  0.6| ± 0.01| ***
| C20:5n-3|  0.9| ± 0.02|  0.6| ± 0.03|  0.6| ± 0.001|  0.5| ± 0.02|  0.05| ± 0.001|  0.03| ± 0.01| *|
| C22:5n-3|  0.3| ± 0.04|  0.1| ± 0.01| ***|  0.2| ± 0.02|  0.1| ± 0.001| ***|  0.2| ± 0.02|  0.1| ± 0.001| ***
| C22:6n-3|  0.33| ± 0.03|  0.08| ± 0.02| ***|  0.16| ± 0.02|  0.05| ± 0.01| ***|  0.15| ± 0.01|  0.05| ± 0.01| ***
| ∑n-3|  3.2| ± 0.11|  1.1| ± 0.05| ***|  2.7| ± 0.10|  0.9| ± 0.04| ***|  2.4| ± 0.10|  0.7| ± 0.02| ***
| C18:2n-6| 39.1| ± 0.68| 22.9| ± 0.17| ***| 39.7| ± 0.44| 22.4| ± 0.21| ***| 41.5| ± 0.61| 21.9| ± 0.11| ***
| C18:3n-6|  0.11| ± 0.01|  0.04| ± 0.01| ***|  0.08| ± 0.01|  0.02| ± 0.01| ***|  0.07| ± 0.01|  0.01| ± 0.00| **|
| C20:3n-6|  0.20| ± 0.02|  0.07| ± 0.01| ***|  0.14| ± 0.01|  0.08| ± 0.01| **|  0.11| ± 0.01|  0.05| ± 0.01| **|
| C20:4n-6|  1.6| ± 0.12|  0.5| ± 0.08| ***|  1.0| ± 0.09|  0.3| ± 0.04| ***|  0.8| ± 0.03|  0.3| ± 0.02| ***
| C22:4n-6|  0.4| ± 0.03|  0.1| ± 0.03| ***|  0.3| ± 0.03|  0.1| ± 0.03| **|  0.3| ± 0.02|  0.1| ± 0.02| ***
| C22:5n-6|  0.09| ± 0.01|  0.04| ± 0.01|  *|  0.06| ± 0.01|  0.04| ± 0.01|  0.05| ± 0.01|  0.02| ± 0.01| *
| ∑n-6| 41.5| ± 0.67| 23.7| ± 0.09| ***| 41.2| ± 0.43| 22.9| ± 0.17| ***| 42.8| ± 0.61| 22.4| ± 0.11| ***
| ∑PUFA| 44.6| ± 0.75| 24.7| ± 0.12| ***| 43.9| ± 0.49| 23.8| ± 0.20| ***| 45.1| ± 0.69| 23.1| ± 0.13| ***
| ∑n-6/∑n-3| 13.1| ± 0.35| 21.9| ± 0.89| ***| 15.3| ± 0.47| 26.9| ± 1.16| ***| 18.2| ± 0.59| 30.0| ± 0.81| ***
| ∑SFA/∑PUFA| 0.5| ± 0.01| 1.2| ± 0.01| ***| 0.6| ± 0.01| 1.2| ± 0.01| ***| 0.5| ± 0.02| 1.2| ± 0.01| ***

RET: retroperitoneal white adipose tissue; EPI: epididymal white adipose tissue; MES: mesenteric white adipose tissue; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ND: not detected. Data are expressed as mean ± SEM. P < 0.05 *; P < 0.01 **; P < 0.001 ***.
Figure 1

- Food intake (g/100g/24h)
- Energy intake (Kcal/100g/24h)
- Body weight (g)

Control vs. High-fat diets over 8 weeks.

* p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 2

Food intake (g)

0-12 hours

12-24 hours

0-24 hours

C

HF

C

HF

C

HF

group

insulin

hours
Figure 3

**Graphs and Bar Charts:**

1. **IR/α-tubulin**
   - Comparison between C and HF conditions.
   - Significant differences indicated by asterisks (*).

2. **pIR/α-tubulin**
   - Comparison among C-, C+, HF-, and HF+ conditions.
   - Significant differences indicated by #.

3. **Akt/α-tubulin**
   - Comparison between C and HF conditions.
   - Significant differences indicated by asterisks (*).

4. **pAkt/α-tubulin**
   - Comparison among C-, C+, HF-, and HF+ conditions.
   - Significant differences indicated by ##.
References


