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Original

Visceral adiposity influences glucose and glycogen metabolism in control and hyperlipidic-fed animals

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Abstract

Introduction: **Evidences suggest that fat intake, visceral obesity and intracellular lipids are related to insulin impairment.**

Objective: **The objective of the present paper was correlate visceral obesity and metabolic alterations in control (CTR) and hyperlipidic cafeteria diet (CFT) fed animals.**

Methods: **After 6 months of diet treatment, liver and muscle of the male rats were utilized to determined glucose uptake and glycogen metabolism after administration of 0.4I U/kg insulin in vivo, and correlate the visceral adiposity to these two parameters.**

Results: **Ample range of physiologic answers to body composition in metabolic profile of the both diets was found. No differences were found in glycemia and triacylglycerol after insulin action in both groups, however CFT group accumulated higher adiposity, mostly visceral fat, and showed lower glycogen content in the liver. We also found an inverse correlation between visceral adiposity and glucose uptake and a decrease of the glycogen synthase active form in the liver. CTR animals demonstrated an inverse correlation between glucose uptake and visceral adiposity in the muscle.**

Discussion and conclusion: **It was observed a variability of metabolic alterations in animals which can be related to degree of accumulation of abdominal adiposity and ingestion of diet fats. Further studies will be required to clarify the reasons for the observed liver alterations in CFT and muscle alterations in CTR animals.**

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Key words: *Insulin impairment. Visceral adiposity. Glucose uptake. Glycogen metabolism.*

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LA ADIPOSIDAD VISCERAL INFLUYE EN EL METABOLISMO DE LA GLUCOSA Y EL GLUCÓGENO EN ANIMALES CONTROL Y CON ALIMENTACIÓN HIPERLIPÍDICA

Resumen

Introducción: **Las evidencias sugieren que la ingesta de grasas, obesidad visceral y lípidos intracelulares están relacionados con resistencia a la acción de la insulina.**

Objetivo: **El objetivo del presente trabajo fue correlacionar la obesidad visceral con alteraciones metabólicas en los animales controles (CTR) y alimentados con la dieta de cafeteria hiperlipidica (CFT).**

Metodos: **Después de 6 meses de tratamiento con dieta, el hígado y lo musculo esqueletico de los ratones se utilizaron para determinar la captación de glucosa y el metabolismo del glucógeno después de la administración de la insulina 0.4 UI/kg *in vivo* y correlacionar la adiposidad visceral a estos dos parámetros.**

Resultados: **Una amplia gama de respuestas fisiológicas a la composición corporal era encontrado. No se encontraron diferencias en la glucemia y triglicéridos después de la acción de la insulina en ambos grupos, sin embargo CFT grupo acumuló mayor adiposidad, principalmente adiposidad visceral, y mostraron menor contenido de glucógeno en el hígado. También se encontró una correlación inversa entre la adiposidad visceral y la captación de glucosa y una disminución de la forma activa de la enzima glucógeno sintasa en el hígado. Animales CTR demostrado una correlación inversa entre la captación de glucosa y la adiposidad visceral en el músculo.**

Discusión y conclusiones: **Se observó una gran variabilidad de alteraciones metabólicas en los animales que se pueden relacionados con las tasas de acumulación de la adiposidad visceral y la ingestión de grasas dietéticas. Más estudios serán necesarios para aclarar las razones de las alteraciones observadas en el hígado de los animales CFT y las alteraciones musculares en animales CTR.**

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Palabras clave: *Resistencia a la insulina. Adiposidad visceral. Captación de glucosa. Metabolismo del glucógeno.*

Abbreviations

CTR: Control.
CFT: Cafeteria.
WAT: White adipose tissue.
BAT: Brown adipose tissue.
GS: Glycogen synthase.
KRb: Krebs Ringer bicarbonate buffer.
GSI: Active form of the glycogen synthase.
GST: Total form of the glycogen synthase.

Introduction

Obesity is one of the most prevalent and incident diseases worldwide and can lead to insulin resistance, diabetes mellitus type 2 and cardiovascular diseases.[1,2]

Insulin resistance is characterized by inadequate insulin action to produce a normal response in peripheric tissues.[3] Accumulation of lipids and their metabolites, including fatty acyl CoAs, diacylglycerols, and ceramides inside the cells, caused by hyperlipidic diets or by visceral obesity, can damage insulin receptor signal transduction.[4,5] As a consequence it increases hepatic gluconeogenesis and causes glycogen depletion by altering glycogen synthase (GS) enzymatic activity in liver and muscle.[6,7,8]

Rodents are useful experimental models to study the insulin resistance caused by hyperlipidic diets, visceral obesity and genetic factors. Genetically obese mice demonstrating differences in body composition and in insulin action —with or without resistance— after nine months of high-fat diet.[9]

The cafeteria (CFT) diet can mimic the high energy foods that are ingested by human subjects being considered a robust model of obesity and metabolic syndromes. It was demonstrated previously that CFT diet induced inflammation in liver and white and brown adipose tissues.[10]

But not only obese subjects can develop an impairment of insulin action. Lean and overweight subjects can accumulate higher levels of intramyocellular lipids and its metabolites and as a consequence presented a decrease of glucose uptake in myocytes.[5,11] The aim of the present study was to analyze the correlation of visceral adiposity and glucose and glycogen metabolism in muscle and liver of control (CTR) and cafeteria (CFT) feeding animals after six-months of treatment. In addition, the variability of the body composition and physiologic responses involving glucose and glycogen metabolism to CTR and CFT diet was also considered.

Methods

Chemicals

The analytical degree reagents utilized in the experiments were obtained from Merck SA, Porto Alegre, Brazil. 2-Deoxi-1-^{14}C-glucose (55 mCi/mmol) and D-[U-^{14}C] glucose (3.0 mCi/mmol) were purchased from Amersham.

Animals and treatments

Twenty-one-day-old male Wistar rats supplied by the Instituto de Ciências Básicas da Saúde (Universidade Federal do Rio Grande do Sul -UFRGS, Porto Alegre, Brazil), were housed at 22-24° C and controlled humidity (70-80%) under a 12 h artificial light cycle (8 am to 8 pm). The animals were randomly assigned to two different dietary groups during six months: control (CTR) and high fat – cafeteria diet (CFT).

The control animals (n = 9) were fed a standard pelleted diet (Nuvital CR-1 6002–NUVILAB, Curitiba, Parana, Brazil) containing 25.6% of energy as protein, 63.4% of energy as total carbohydrates, and 11% of energy as fat (% of kcal). Water was provided ad libitum.

The CFT group (n = 11) was fed a high-fat diet containing 15% of energy as protein, 60% of energy as total carbohydrates, and 25% of energy as fat (% of kcal). The cafeteria diet was composed of sweet biscuits, bread, french fries, chocolate, fatty ham, banana, liver pate, sausage, fatty cheese, condensed milk, and smoked bacon. All foods of CFT diet and water were offered in natura and ad libitum with the standard diet. Food intake was measured when the animals were four months old and during four weeks. Carbohydrate, lipid, and protein contents of the food constituents were determined by the Instituto de Ciências e Tecnologia de Alimentos at UFRGS.

Special care was taken to limit the number of animals used and their suffering. All animal procedures followed in this study were in accordance with the Principles of Laboratory Animal Care (COBEA-Brazilian College of Animal Experimentation), and the experimental protocol was approved by the UFRGS Animal Care Committee.

Experimental procedure

After six months of treatment, and two days before the experimental procedure, the animals received a canula in a vein through a surgical procedure. One day before the experiment, the animals were submitted to 8 h of fasting and on the day of the experiment the rats were weighed and received 0.4 IU/kg of human insulin. An aliquot of blood was removed from the canula for dosage of fasting glycemia (0 min) and after 16 min of the insulin administration. The blood was centrifuged and the plasma used to measure plasma glucose by an enzymatic glucoseoxidase method (Glucose PAP Liquiform-Kit Labtest Diagnostics). Triacylglycerol was also measured after fasting (0 min) by an enzymatic method according to the manufacturer's instructions (Trinder Liquiform-Kit Labtest Diagnostics).

Both measurements were performed with the help of the Espectrofotometer Ultraspec 2000 (Pharmacia Biotech) using wave lengths of 505 nm and 520 nm for glucose and triacylglycerol, respectively. The values of glycemia and triacylglycerol were expressed as mg/dL.

Body composition

The animals were weighed and measured (length were measured in cm) after submitted to any treatments or diets. After insulin administration, the animals were sacrificed by ether. White (WAT) and brown adipose tissue (BAT) were dissected from abdomen and perilumbar regions, respectively, and weighed to verify the total adipose tissue. Free-fat weight was obtained by the subtraction of the total weight from WAT and BAT. All weights described are expressed in grams (g).

We also calculated the WAT index obtained by the division of weight of WAT per total weight; the BAT index obtained by the division of weight of BAT per total weight; and the free-fat weight index obtained by the division of weight of free-fat mass per total weight. All the indexes are expressed in percentage (%).

After the experiment, the liver and the gastrocnemic muscle were also dissected and divided in three parts. The first was incubated with 2-Deoxi-D-1-^{14}C-Glucose to assess glucose uptake or with D-[U-^{14}C] glucose to verify ^{14}C-glycogen synthesis. The second was used to verify the total glycogen content, and the third was frozen in nitrogen and utilized to assess glycogen synthase (GS) activity.

In vitro experiments: 2-^{14}C-DeoxiGlucose uptake and ^{14}C-glycogen synthesis

To determine glucose uptake and glycogen synthesis *in vitro*, liver and muscle were rapidly removed and placed on a Petri dish containing cold incubation buffer and cut in slices.

To measure [2-Deoxi-1-^{14}C-glucose] glucose uptake, slices (about 200 mg/tube) were incubated in 1 mL Krebs Ringer bicarbonate (KRb) buffer, pH 7.4, at 37° C with constant shaking and containing 0.15 mCi 2-Deoxi-1-^{14}C-glucose. The contents of the tubes were gassed with O_2:CO_2 (95:5, v/v) for 1 min and then closed. The tissues were incubated in a Dubnoff incubator at 37° C for 1h with constant shaking. After incubation, the tissues were withdrawn, rinsed in cold incubation buffer (three times) and blotted with filter paper. The 2-Deoxi-1-^{14}C-glucose was extracted from the tissue and the radioactivity was measured in LKB-Wallac scintillation counter. Glucose uptake was immediately determined, according to Machado et al.[12] Results are expressed as tissue/medium (T/M) ratio, i.e., dpm/mL tissue fluid per dpm/mL of incubation medium.

For ^{14}C-glycogen synthesis, slices (about 200 mg/tube) were then incubated at 37° C for 1 h with constant shaking in 1 ml of Krebs Ringer bicarbonate (KRb) buffer, pH 7.4, equilibrated with O_2:CO_2 (95:5, v/v) for 1 min, in the presence of 0.15 mCi [U-^{14}C]L-glucose and 10 mM of unlabelled glucose. After the incubation, tissue reactions were stopped in an ice bath, and the tissues were washed in cold buffer and dried on filter paper. The tissue proteins were precipitated by addition of 30% trichloroacetic acid and 1N HCl (2:1, v/v). After centrifugation at 600 × g for 10 min, the supernatants (30 µl) were applied on Whatman 3MM strips. The ^{14}C-glycogen was precipitated by 66% ethanol[13] and the radioactivity was measured in LKB-Wallac scintillation counter. Results are expressed in pmol/mg of tissue/ incubation time.

Hepatic and muscle glycogen content in vivo

Hepatic and muscle tissues were frozen at -20º C until ready for analysis. Glycogen was extracted essentially according to Van Handel,[14] and determined as glucose after acid hydrolysis,[15] using rabbit glycogen as standard. Glucose content after hydrolysis was determined by enzymatic glucoseoxidase method (Glucose PAP Liquiform-Kit Labtest) and measured in espectrofotometer (Espectrofotometer Ultraspec 2000-Pharmacia Biotech) using 505 nm wave length. Values were expressed as g% of glycogen.

Glycogen synthase (GS) activity assay

To verify GS activity, liver and muscle were quickly dissected, frozen on liquid nitrogen, and kept at -70º C until ready for analysis. GS activity was measured according to Nuttall and Gannon,[16] but following the modifications described previously.[17] Protein concentration was determined by the method described by Bradford.[18] Enzyme activity was calculated in nmoles of UDP-glucose-U-^{14}C incorporated into glycogen/ milligram of protein/ minute.

Statistical analyzes

Data from the experimental procedures were used to compare the effects of insulin in the serum glucose of the CTR versus CFT diets by two-way ANOVA followed by Tukey's least significance test. Student's t-test was used to verify statistical differences between glycemia at fasting (0 min) and 16 min after insulin administration and to compare the glycemia between CTR and CFT groups for each time (0 or 16 min).

Differences in food intake along the four weeks were analyzed by one-way ANOVA and next by Tukey's least significance test. The other experimental conditions between the two groups were compared using

Table I
Body composition and serum parameters of control (CTR) and cafeteria-diet-fed (CFT) animal groups after six months

	CTR group [n]	*CFT group [n]*	*P significance*
Body composition			
Length (cm)	252.22 ± 14.6	259.36 ± 19.43	p > 0.05
Total weight (g)	425.06 ± 42.99[9]	534.06 ± 44.93[11][a]	p ≤ 0.001
Total fat weight (g)	11.13 ± 2.73[9]	39.44 ± 9.64[11][a]	p ≤ 0.001
BAT index (%)	0.07 ± 0.02[9]	0.11 ± 0.03[11][b]	p = 0.002
WAT index (%)	2.49 ± 0.65[9]	7.15 ± 1.69[11][a]	p ≤ 0.001
Free-fat mass index (%)	97.44 ± 0.63[9]	92.74 ± 1.70[11][a]	p ≤ 0.001
Serum parameters			
Fasting glycemia (0 min)	89.71 ± 12.01[8]	77.92 ± 28.82[9]	p > 0.05
After 16 min of insulin administration	44.85 ± 5.76[5][c]	42.27 ± 10.32 [7][d]	p > 0.05
Triacylglycerol	39.93 ± 12.0[7]	42.89 ± 17.52[8]	p = 0.712

P significance demonstrates the statistical difference between both groups. Statistical difference in Glycemia from fasting: cp < 0.001 and dp = 0.008. The total and fat mass weights are expressed in grams (g), the indexes in percentage (%); and glucose and fasting plasma triacylglycerol as mg/dL. The value for each n is represented in each line.
BAT: Brown adipose tissue; WAT: White adipose tissue.

Student's t-test. Results are expressed as mean ± S.D. (standard deviation). For correlations between total fat mass weight, visceral adiposity (WAT weight), or perilumbar adiposity (BAT weight) and glucose uptake or ^{14}C-glycogen synthesis, simple linear regression and the Pearson correlation were used. The level of significance was set at $p < 0.05$.

The Multivariate Cluster analyzes (Euclidean distance) was performed to evaluate the heterogenic characteristics of animals from both groups utilizing the total body weight, WAT and BAT weight as variables.

Results

Food intake

Food intake of the CFT rats did not show statistical difference during the 4 weeks of measurement (data not shown; p = 0.679). However, the food ingestion of CFT animals had higher fat content (46.41%) and lower protein and carbohydrate content (14.04 and 39.45%, respectively) than the diet provided to the CTR animals.

Body composition and serum parameters

Body composition and indexes demonstrated an increase in total fat mass content with visceral (WAT) and perilumbar (BAT) fat accumulation in animals fed with CFT diet (table I).

The profile of glycemia and triacylglycerol observed in the serum was not different between CTR and CFT groups (table I).

Glucose and glycogen metabolism: correlation to visceral adiposity

No difference in 2-^{14}C-DeoxiGlucose uptake and ^{14}C-glycogen synthesis *in vitro* was observed between the CTR and CFT groups (fig. 1). However, simple linear regression demonstrated an inverse correlation between 2-^{14}C-DeoxiGlucose uptake and total fat mass weight (r^2 = -0.79, p = 0.03), and between 2-^{14}C-DeoxiGlucose uptake and WAT weight (r^2 = -0.80, p = 0.033) in the liver from CFT group, but not in muscle (r^2 = -0.43, p = 0.32 and r^2 = -0.43, p = 0.326, respectively). Corroborating these results, glycogen content of liver from CFT rats was decreased after insulin administration (p = 0.021) (table II).

Table II
Muscle and liver glycogen concentration after insulin administration to control (CTR) and cafeteria-diet-fed (CFT) animals

	CTR [n]	*CFT [n]*
Muscle	0.045 ± 0.03 [4]	0.064 ± 0.1 [8]
Liver	0.523 ± 0.31 [5]	0.179 ± 0.15 [7]*

*Statistical difference from the control (p = 0.021). Results are expressed as g%. The value for each *n* is represented in each line.

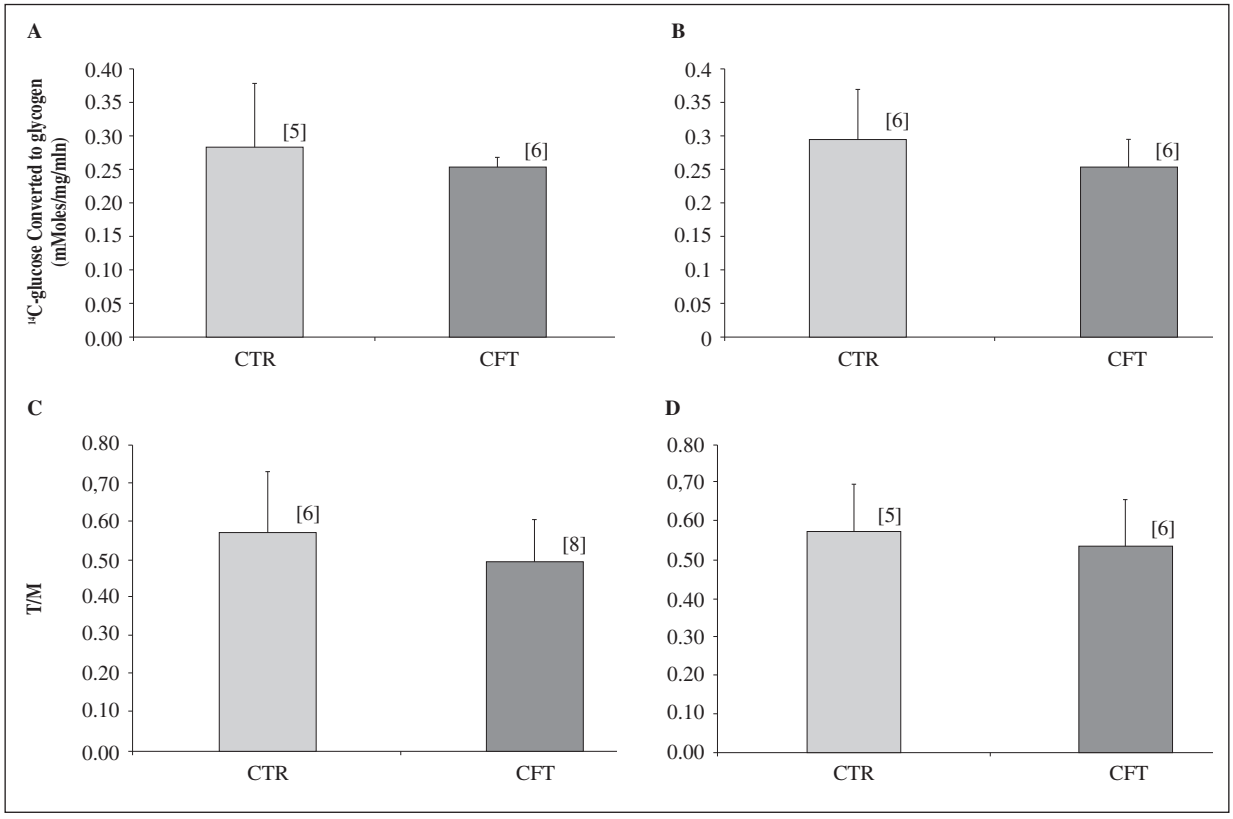

Fig. 1.—Effect of 0.4 IU/kg insulin on 14C-glycogen synthesis and 2-14C-DeoxiGlucose uptake in control (CTR) and cafeteria (CFT) fed animals. A) 14C-glycogen synthesis in liver. B) 14C-glycogen synthesis in muscle. C) 2-14C-DeoxiGlucose uptake in liver. 1D: 2-14C-DeoxiGlucose uptake in muscle. There was no difference between the groups tested ($p > 0.05$). The value for each n is represented in each figure. Results of A and B are expressed as pmol/mg of tissue/incubation time, and C and D as tissue/medium (T/M) ratio (that is, dpm/ml tissue fluid per dpm/ml incubation medium). See the experimental procedure section for details.

For the CTR group we found an inverse correlation between 2-^{14}C-DeoxiGlucose uptake, and total fat mass weight ($r^2 = -0.89$, $p = 0.017$), and 2-^{14}C-DeoxiGlucose uptake and WAT weight ($r^2 = -0.89$, $p = 0.018$) in muscle. This data indicates that after six months of treatment, rats with higher visceral fat weight have impaired muscle glucose uptake, as demonstrated by insulin administration. Results for the CTR group liver revealed no statistical significance when same correlations were analyzed (data not shown).

No correlation between ^{14}C-glycogen synthesis *in vitro* and total fat mass weight or WAT weight was observed (data not shown). The correlation between ^{14}C-glycogen synthesis or 2-^{14}C-DeoxiGlucose uptake and BAT was also tested but no difference was revealed in liver and muscle (data not shown).

Glycogen synthase (GS) activity

The measured GS activity did not demonstrate any difference between CTR and CFT (figure 2). However, the active form of the enzyme GS (GSI) was lower than that of the total form (GST) in the liver of CFT and in CTR even after insulin administration (fig. 2).

Multivariate cluster analyzes

Multivariate Cluster analyzes were performed to determine the range of physiologic response regarding the body composition profile shown by the animals from both diet groups (fig. 3). The CTR group showed heterogenic responses to the diet as demonstrated by body composition analyzes. The response to CFT diet also varied among the animals, although the observed response was more severe than that found for the CTR group. These finds can be related to the heterogenic metabolic profiles described in the present paper.

Discussion

Our study attempted to determine the effect of visceral obesity in the parameters of glucose and glycogen metabolism in muscle and liver of six-months old rats. We found that some parameters such as glycemia and tryacylglicerol were not significantly different between the CTR and CFT groups. In part, the no significant responses can be explicated by heterogenic responses observed from clustered data of body composition. When data were distributed correlating glucose uptake *in vitro* and adiposity (mostly visceral

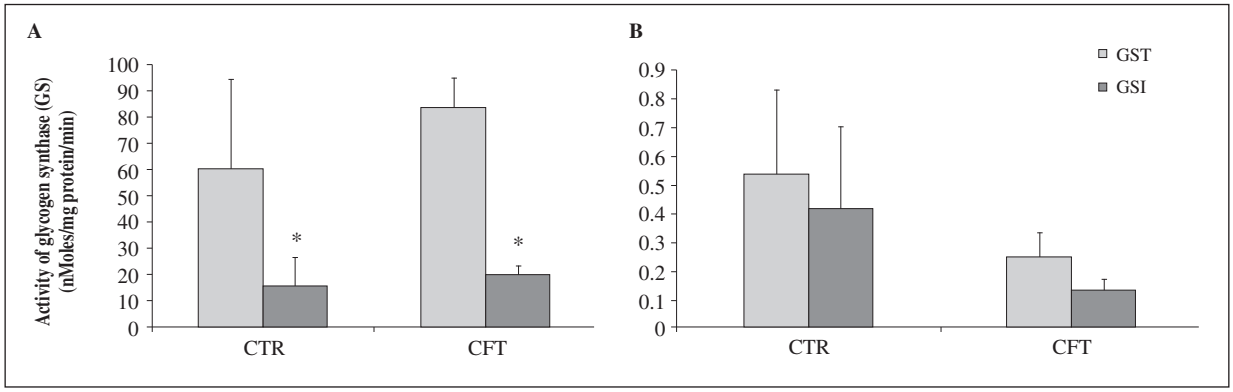

*Fig. 2.—Liver and muscle glycogen synthase (GS) activity in control (CTR) and cafeteria-diet-fed (CFT) animals. A) liver and B) muscle. There was no difference between the groups tested ($p > 0.05$). *Indicates difference between GST and GSI in liver of CTR ($p < 0.001$) and CTR ($p < 0.046$). GST: Total form of GS; GSI: Active form of GS. GS activity is expressed as nMoles/mg of protein/min. The n = 4, for every dosage (duplicate analyzes from each animal).*

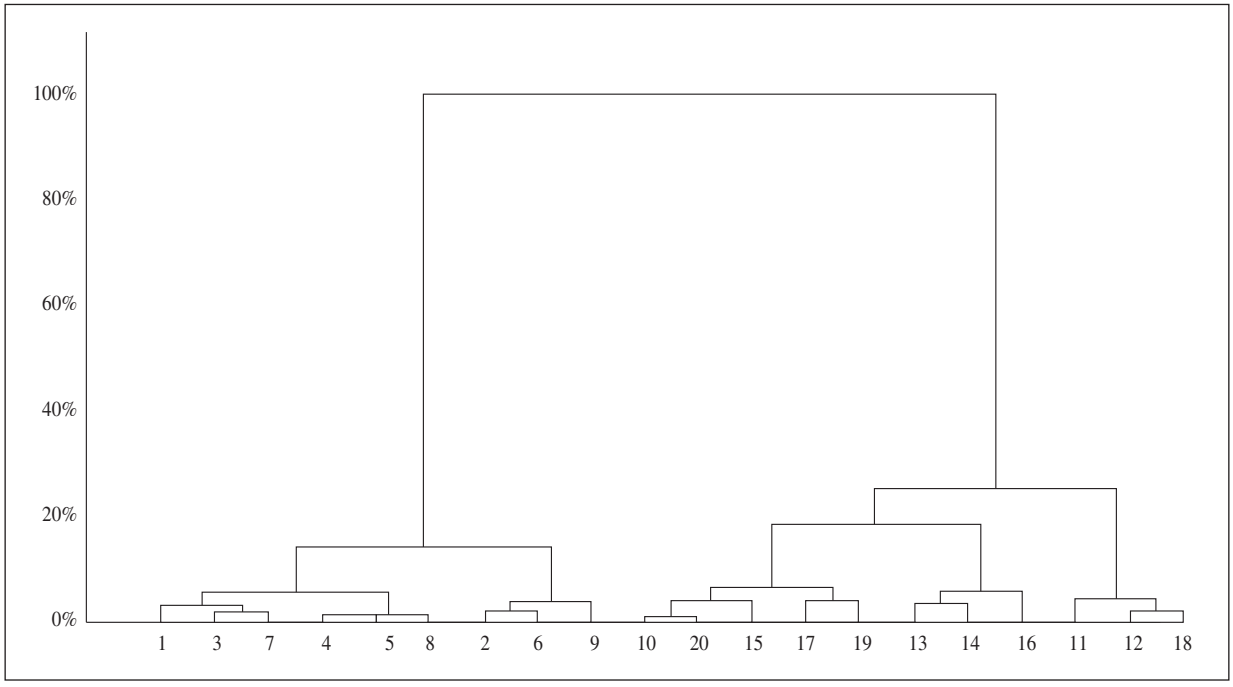

Fig. 3.—Multivariate Cluster analyzes of body composition of animals from control (CTR) and cafeteria-diet-fed (CFT) groups. CTR group animals: 1 to 9; CFT group animals: 10 to 20.Body weight, White Adipose Tissue (WAT), and Brown adipose Tissue (BAT) weight were used as variables.

adiposity) differences were observed, demonstrating variations in the animal responses inside the same group. In addition, GSI activity was decreased in the liver of CTR and CFT animals, but not in muscle, demonstrating alterations in glycogen metabolism related to visceral obesity.

In the CFT diet, the caloric intake was high-fat, low-carbohydrate, and low-protein when compared to CTR animals, demonstrating a preference for fat-intake by the rats that corroborated to previous studies.[10,19,20] It was also observed that CFT-fed rats gained total fat mass, mostly visceral, when compared to CTR animals. Visceral adiposity is related to intrahepatic triacylglycerol and other lipids contents and can be responsible for many of the metabolic abnormalities associated with abdominal obesity and insulin resistance.[6,10,21] It was not observed any negative influence of CFT diet in the growing process of the animals. Multivariate cluster analyzes of body composition data demonstrated a heterogenic response to the diet in both the CTR and CFT groups, however it was more pronounced in the CFT group. The hyperlipidic diet allowed animals to develop a wide range of weight gain and, as a consequence, heterogenic metabolic profiles. The CFT animals that accumulated more visceral adiposity demonstrated less glucose uptake in liver.

Serum glycemia and triacylglycerol was not different between CFT-fed and CTR-fed animals. Other

researchers have reported similar results of plasma lipids, although their studies reported an increase in other lipids, i.e., cholesterol and nonesterified fatty acids.[22,23] The serum profile can be also related to the heterogenic physiologic responses of animals of both diet treatments as observed in cluster analyzes.

Data dispersion indicated a great and strong variability in the responses by the CFT group. It was not possible to observe a direct effect of CFT diet on plasma glucose, 2-^{14}C-DeoxiGlucose uptake, and ^{14}C-glycogen synthesis after insulin administration when compared to the CTR group. However, when the results of 2-^{14}C-DeoxiGlucose uptake were correlated to the total fat and visceral adiposity, they revealed that this progressive accumulation negatively altered glucose uptake in the liver. This strong effect was demonstrated by the decreased glycogen levels found in the livers of CFT-fed animals. Decrease of glycogen levels in the liver are related to lipid accumulation inside the cells that decrease of insulin action.[6,7] Consistent with this notion, our results showed that glycogen levels in the liver decrease following insulin administration.

The visceral adiposity may be related to the great fat ingestion by the CFT groups and the first tissue that metabolizes the lipids is the liver. Liver is a key player in the control of whole body energy metabolism because of its ability to regulate glucose and fatty acid metabolism and to act as a systemic lipid buffer during periods of high lipid influx (24; 25) and higher waist circumference is correlated to low levels of HDL-c (26). The CFT showed clear alterations of insulin signaling that can be related to accumulation of lipids and metabolites as fatty acyl CoAs, diacylglycerols, and ceramides caused by hyperlipidic diets and visceral obesity (4).

We also observed a significant decrease in GSI activity in the liver but not in muscle of the CFT group. Previous studies reported that glycogen depletion and altered GS enzymatic activity are characteristic features of insulin impairment in insulin-metabolic dependent tissues.[6,7,8]

In the CTR group an inverse correlation was observed between 2-^{14}C-DeoxiGlucose uptake in the muscle and body adiposity, mostly visceral fat. This was observed when the animal accumulated total fat mass and WAT weight, although the values of both weights were significantly lower compared to CFT-fed animals.

The CTR group showed some variability responses to control diet, as clustered data can demonstrate. Previous reports revealed that lean and overweight subjects can present an inverse correlation between glucose uptake and intramyocellular lipid content related to impairment of insulin action.[5,11]

In the CTR group we also observed an interesting decrease of GSI activity in the liver, in the presence of insulin administration however no negative impact in glycogen content was observed.

Conclusion

The accumulation of intracellular lipids and the impairment of insulin action are possible answers for the observed differences in the metabolic state of the CTR and CFT animals. In addition, multivariate cluster demonstrated the variable range of physiologic responses to the diets as observed by the body composition profiles of both experimental groups. In this study we reveal that in the case of the CFT group primary tissue for lipid metabolism and accumulation seems to be the liver. Because the CTR group was fed with a low-fat diet, the animals may respond accumulating lipids in their muscle however the reasons for that need to be clarify. Our results indicate a clear correlation of visceral adiposity and impaired glucose uptake that is influenced by the diet, the degree of fat accumulates in the tissues and the degree of overweight and/or obesity. Further studies are required to unveil the endocrine and molecular mechanisms which account for the differences found for the two groups of animals studied herein.

Competing interests

The authors declare that they have no competing interests and did not receive any funding.

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Author's contributions

Danielle Kaiser de Souza wrote the present paper and did all the experiments. Fabiana A. de Souza, and Signorá Peres Konrad contributed significantly to all the experimental procedures. Luciano Stürmer de Fraga helped us in the GS activity measurement.

Adriane Belló-Klein and Roselis Silveira Martins da Silva provided materials and intellectual support for the experiments. Luiz Carlos R. Kucharski was the supervising professor of the present paper.

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