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CONSUMO DE DIETA HIPERPALATÁVEL, ALTERAÇÕES METABÓLICAS E COMPORTAMENTAIS: UM MODELO DE OBESIDADE EM RATOS E SUAS CONSEQUÊNCIAS

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“Tudo posso Naquele que me fortalece”

Filipenses 4:13

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SUMÁRIO

RESUMO

A obesidade é a maior síndrome do século XXI, alcançando proporções epidêmicas em todo mundo. Muitas são as causas para o seu desenvolvimento, porém a mais importante, provavelmente, é o consumo aumentado e a grande disponibilidade de alimentos altamente palatáveis, ricos em açúcar e gordura. A obesidade e o consumo destes alimentos são associados ao aparecimento de doenças como hipertensão, diabetes tipo 2 e doenças cardiovasculares. A resistência à insulina, a diminuição dos níveis de adiponectina e do óxido nítrico circulantes são fatores de riscos importantes para o surgimento de doenças cardiovasculares. Além disso, a diminuição dos níveis séricos de adenosina também está associada com o aparecimento de doenças cardiovasculares e que esta diminuição pode ser influenciada por alguns fatores ambientais. A hidrólise dos nucleotídeos da adenina, ATP, ADP e AMP, é uma das formas de manter os níveis de adenosina circulantes e por isso a atividade das enzimas ectonucleotidases, que regulam esta hidrólise, é importante para homeostase cardiovascular. Em face disto, tivemos por objetivo verificar a atividade destas enzimas em um modelo de indução de obesidade por meio de dieta hiperpalatável, além de verificar as demais alterações na composição corporal e no metabolismo da glicose, dos lipídios séricos, nos níveis de insulina, adiponectina e de óxido nítrico, focando nos possíveis efeitos dessas alterações no sistema caridiovascular. Nossos resultados indicaram que a obesidade induzida pela dieta promove um perfil de alterações semelhante a diabete mellitus tipo 2, acompanhada de diminuição dos níveis de óxido nítrico e de diminuição acentuada da atividade das ectonucleotidases, promovendo um ambiente pró-aterogênico, mesmo sem alteração dos níveis de insulina e adiponectina. Além das alterações no metabolismo, a obesidade e o consumo de alimentos altamente palatáveis está associado com alterações cognitivas e comportamentais, como ansiedade, depressão e memória. Sabe-se também que estes alimentos geram um padrão de adição nos centros neurais, que pode estar relacionado com doenças psiquiátricas como transtornos de humor e ansiedade, e muitos estudos populacionais mostram que o estresse oxidativo também pode ser associado à estes transtornos. Em indivíduos intolerantes à glicose, a hiperglicemia é a maior fonte de produção de radicais livres e em função disto, decidimos testar nossos animais submetidos à dieta hiperpalatável para avaliar possíveis alterações comportamentais e após os testes, medir o estresse oxidativo no córtex frontal e hipocampo destes animais. Nossos resultados mostraram que os animais que receberam a dieta hiperpalatável são mais ansiosos dos que receberam a dieta controle, porém sem alterações de locomoção ou memória. Além disso, os mesmos apresentam maior dano oxidativo nas proteínas do córtex frontal, uma área muito ligada ao comportamento emocional de ansiedade e medo, mas sem alterações no hipocampo. Em função do exposto, propomos que além de promover alterações no metabolismo da glicose e aumentar o risco de doenças cardiovasculares, a obesidade promovida pelo consumo de uma dieta hiperpalatável aumenta a ansiedade e a oxidação de proteínas no córtex frontal, afetando também o sistema nervoso central e o comportamento emocional.

ABSTRACT

Obesity is the major syndrome of 21st century, reaching epidemic proportions worldwide. There are many causes for obesity but the most important is probably the overeating and the ready availability of food rich in fat and sugar. Obesity and the consumption of this type of food are associated to hypertension, type 2 diabetes and cardiovascular diseases as consequences. Insulin resistance, decreased adiponectin levels and nitric oxide circulating are strong risk factors to development of cardiovascular diseases. Besides, decreased of serum adenosine levels are also associated to cardiovascular diseases and those leveles can be affected by environmental factors. Adenine nucleotides hydrolysis ATP, ADP and AMP is one way to keep the circulating adenosine levels by ectonucleotidases activity, which regulates this hydrolysis. For that reason, this enzymes activity is important for cardiovascular homeostases. Based on this, the aim of our study was to verify the ectonucleotidases activity in a model of obesity induction through a highly palatable diet consumption. We also intent to verify another alterations in body composition, glucose metabolism, serum lipids, insulin, adiponectin and nitric oxide levels, focusing in possible effects of these alterations in cardiovascular system. Our results showed that obesity induced by this diet provokes alterations type 2 diabetes like, reduced levels of nitric oxide and accentuated decreased of ectonucleotidases activity, promoting a vascular pro-atherogenic environment, even without alterations in insulin and adiponectin levels. Besides metabolic alterations, obesity and the consumption of palatable foods are associated to cognition and behavioral alterations as anxiety, depression and memory capacity. Is well known that in brain circuits palatable foods promote an addiction profile, which may be related to psychiatry disorders like mood and anxiety. Populational studies showed that oxidative stress also can be associated to these alterations. As glucose intolerace is the main source of free radicals production in glucose intolerant individuals, we decided to test our animals submitted to a highly palatable diet to search for possible behavioral alterations. After behavioral tests, we decided to measure oxidative stress in frontal cortex and hippocampus of the same animals. This other part of our results showed that the animals submitted to a highly palatable diet are more anxious than the animals submitted to a standard diet, but there were no alterations in locomotion and memory capacity. The same animals also presented higher oxidative protein damage in frontal cortex, an important brain structure involved in behavioral regulation and is part of several well-defined anxiety and fear-related circuits in the forebrain, however no alterations was observed in the hippocampus. Therefore, we propose that beyond alterations in glucose metabolism and increasing cardiovascular diseases risk, obesity induced by consumption of a highly palatable diet increases anxiety and frontal cortex protein oxidation, also affecting central nervous system and emotional behavioral.

LISTA DE ABREVIATURAS

ATP – adenosina trifosfato

ADP – adenosina difosfato

AMP – adenosina monofosfato

TAG – triacilglicerol

NO – óxido nítrico

RL – radicais livres

SM – Síndrome Metabólica

DM2 – Diabete Mellitus tipo 2

ENOS – óxido nítrico sintase endotelial

IRS1- substrato receptor de insulina 1

1. INTRODUÇÃO

1.1.Obesidade e dieta hiperpalatável

A obesidade pode ser considerada uma das principais síndromes do século XXI, estando envolvida na etiologia de uma série de doenças como resistência à insulina, diabetes, hipertensão e cardiopatias (Unger and Orci, 2001; Dube et al, 2005). Durante o século XX uma mudança nos padrões de disponibilidade de calorias tomou conta de países ocidentais. Contribuem para isso, os avanços tecnológicos do mundo moderno, que tornaram o estilo de vida sedentário, e o maior acesso aos alimentos, que possibilita uma maior ingestão calórica diária. Freqüentemente, muitos desses alimentos não são os mais nutritivos e saudáveis, mas sim, os mais "palatáveis" e baratos (Chakravarthy and Booth, 2004). Embora o alto consumo de uma dieta rica em lipídios, carboidratos simples e a diminuição da atividade física seja facilmente capaz de induzir a obesidade, fatores genéticos também precisam ser levados em conta, mesmo não justificando os níveis epidêmicos de obesos na população mundial (Froguel et al., 2000; Saper et al., 2002).

Os alimentos hiperpalatáveis são capazes de estimular o consumo alimentar e promover mudanças no metabolismo por alterarem a razão fisiológica entre moléculas orexígenas/ anorexígenas, muitas das quais estão relacionadas com a utilização da glicose, a oxidação dos ácidos graxos, o gasto energético, além do próprio mecanismo de saciedade em si, as quais são bases da obesidade e de patologias relacionadas (Erlanson-Albertsson, 2005). Como os mecanismos de homeostasia que tentam controlar as conseqüências da "superalimentação" foram incapazes de compensá-la, em função do aumento crônico no

balanço calórico, as doenças relacionadas à superalimentação tornaram-se incrivelmente prevalentes (Neel, 1999).

Em indivíduos obesos alterações como intolerância à glicose, resistência periférica à insulina, hipertensão, dislipidemias e aterosclerose estão freqüentemente presentes como comorbidades (Takahashi et al., 2007; Wassink et al., 2007). O conjunto destes fatores é denominado Síndrome Metabólica (SM) e na maior parte dos casos, esta evolui para o Diabete Mellitus tipo 2 ou para doença cardiovascular ou, em alguns casos para ambos (Naderali et al., 2004). Portanto, o tipo de dieta pode ser considerado um fator determinante para a formação do percentual de gordura bem como para o aparecimento ou a prevenção das patologias associadas à obesidade (Cnop et al., 2003).

1.2. Resistência à insulina e Diabete Mellitus tipo 2

O aumento de gordura corporal, especialmente da gordura visceral, promove o aumento de ácidos graxos livres e acúmulo dos mesmos em tecidos que não sejam o adiposo, como fígado, músculo esquelético e até mesmo nos vasos sangüíneos (Arner et al., 2005; Yudkin et al., 2005). Ao acúmulo de ácidos graxos livres em tecidos não adiposos foi atribuído o termo de lipotoxicidade e acredita-se que esta é a causa em comum das complicações da obesidade, resistência à insulina, diabetes tipo 2 e doenças cardiovasculares (Unger and Orci, 2001). Esses ácidos graxos acumulados inibem o metabolismo de carboidratos nestes tecidos via competição de substrato e assim impossibilitam as ações fisiológicas da insulina, causando resistência periférica à mesma, promovendo hiperglicemia (Yudkin et al., 2005). À hiperglicemia promovida e à suas conseqüências foi dado o nome de glicotoxicidade, caracterizadas pela alta capacidade de

produção de radicais livres e alteração na estrutura e função de proteínas, lipídios, citocinas e até mesmo do DNA das células lesadas, sendo as células endoteliais dos capilares , as da retina, dos glomérulos renais e dos nervos periféricos as mais suscetíveis à lesões (Brownlee, 2005). O quadro de resistência periférica à insulina, por glicotoxicidade e/ou lipotoxicidade, acrescido da diminuição da secreção pancreática deste hormônio caracteriza a instalação da diabete mellitus tipo 2 (DM2) (Mc Garry, 2002). O DM2 tem por conseqüências hipertensão, dislipidemias, esteatose hepática, complicações cardiovasculares e ateroscleróticas, retinopatia, nefropatia, neuropatia periférica e até mesmo desordens psiquiátricas (Pinhas-Hamiel and Zeitler, 2007).

O aumento do tecido adiposo é um fator determinante para todas estas alterações pois além de desregular o metabolismo da glicose e dos ácidos graxos por meio da lipotoxicidade e da glicotoxicidade, gerando resistência à insulina, ainda promove alterações nas citocinas secretadas pelo mesmo. O tecido adiposo não é mais visto atualmente somente por sua função energética, mas sim como um órgão ativo em secreções que regulam desde à sensibilidade à insulina até o controle do peso corporal, saciedade e resposta inflamatória (Prins, 2002). Dentre as citocinas secretadas por este tecido, as mais relacionadas com a síndrome metabólica e com o DM2 são a adiponectina e a leptina, por exercerem ações pró-insulina e também por aumentarem a oxidação de lipídios (Park et al., 2003; Gil-Campos et al., 2004). Os níveis de adiponectina tem sido utilizados atualmente como preditores da síndrome metabólica e de doenças cardiovasculares, em função de seu papel antiaterogênico, protetor do sistema cardiovascular e por estarem diminuídos com o aumento da gordura corporal (Rothenbacher et al., 2005).

Além de levar ao acúmulo de lipídios no fígado e no músculo, a inabilidade da insulina também está associada à diminuição de uma vasodilatação-dependente do endotélio vascular, em resposta a vários vasodilatadores afetados pela obesidade, inclusive a própria insulina. A diminuição desta resposta é um dos fatores que viabiliza disfunções endoteliais em indivíduos insulino-resistentes ou diabéticos (Yudkin et al., 2005).

1.3. Doenças cardiovasculares

Embora os estudos tenham mostrado que as causas clássicas para doenças cardiovasculares (tabagismo, hipertensão e dislipidemia) tenham diminuído nos últimos 25 anos, o aumento da obesidade, do DM2 e da resistência à insulina manteve a prevalência destas doenças aumentada como antes (Smith, 2007). A aterosclerose é a causa de morte de ¾ dos pacientes que vão à óbito por doenças cardiovasculares (Cheng, 2005).

Os mecanismos fisiopatológicos que aumentam o risco de doenças cardiovasculares em indivíduos obesos e com resistência à insulina são a hipertensão, os estados pró-inflamatório e pró-trombótico, dislipidemias, além da hiperglicemia, que gera metabólitos pró-oxidantes (Rader, 2007). Estes fatores associados promovem disfunção endotelial, seja por maior resposta inflamatória, agregação plaquetária, oxidação de LDL, vasoconstrição ou todos juntos.

A resistência à insulina afeta também o endotélio vascular por diminuir a produção de óxido nítrico, que atua como vasodilatador endógeno. A ativação da enzima óxido nítrico sintase (e-NOS) ocorre por meio da ativação da cascata insulínica e em resposta à mesma, ocorre diminuição dos níveis de endotelina, que atua como vasoconstrictor (Yudkin et al., 2005). O aumento na ingestão calórica também afeta esse sistema, uma vez que em

situações de ingestão excessiva de calorias é formado um depósito de gordura local nos vasos sangüíneos, com funções vasoregulatórias específicas, secretando citocinas e regulando as funções deste endotélio, desde atividade enzimática até a transdução de sinais (Mazurek et al., 2003).

Recentemente a diminuição dos níveis de adenosina vem sendo muito associada à um maior risco de doenças cardiovasculares, principalmente por desempenhar um efeito vasodilatador e de melhora do fluxo sangüíneo durante situações de hipóxia (Berne, 1963). Além disso, ela inibe a agregação plaquetária, a proliferação de células musculares lisas e a adesão de neutrófilos ao endotélio vascular, sendo todos estes fatores associados a fisiopatologia de doenças cardiovasculares (Mubgawa et al., 1996; Ralevick and Burnstock, 2003). Sua liberação na corrente sangüínea pode ocorrer por secreção celular ou pela degradação dos nucleotídeos da adenina extracelulares ATP, ADP E AMP (Latini e Pedata, 2001). Esses nucleotídeos podem ser degradados por nucleotidases, que estão localizadas em superfícies celulares e solúveis nos meios extracelulares, sendo o endotélio vascular um de seus principais sítios de localização (Sarkis et al., 1995; Zimmermann et al., 2001). Alguns fatores ambientais podem diminuir a atividade destas enzimas, como o uso de imunosupressores e a hiperhomocisteinemia, fatores estes independentes para doenças cardiovasculares (Chen et al., 2002; Koymada et al., 1996). Em função disto, tem-se postulado que controle das atividades dessas nucleotidases são importantes para impedir processos de formação de trombos vasculares pela formação e manutenção do equilíbrio dos níveis da adenosina, mantendo sua homestase no sistema vascular.

1.4. Alimentos palatáveis, obesidade e comportamento

A obesidade pelo consumo de alimentos palatáveis, tem sido investigada não só por suas alterações metabólicas, mas também por alterações comportamentais apresentadas por indivíduos acima do peso. Estudos experimentais têm focado suas investigações em mecanismos moleculares e alterações comportamentais específicas por consumo de dietas ricas em açúcar e gordura, promovendo alterações nas células neuronais (Molteni et al, 2002, 2004).

A obesidade por consumo de alimentos altamente palatáveis está associada com mecanismos de recompensa, promovendo uma resposta de adaptação e gerando padrões de adição nos centros neurais estimulados por ela (Colantuani et al., 2002; Erlanson-Albertsson, 2005). Estudos populacionais com pacientes sobrepreso e obesos mostraram que existe uma prevalência maior de doenças psiquiátricas dentre estes, especialmente ansiedade, depressão e transtornos de humor (Chakravarthy et al., 2004, Simon GE, 2006; Becker et al., 2001; Teegarden and Bale, 2007).

A ansiedade é uma das desordens psiquiátricas mais prevalentes atualmente (Gingrich,2005) e recentemente foi associada ao stress oxidativo, por meio de estudos que mostraram que genes envolvidos na produção de RL e nas defesas antioxidantes poderiam modular para mais ou para menos o nível de ansiedade em modelos animais, dependendo do maior ou menor nível de estresse gerado (Hovatta et al., 2005; Berry et al., 2007).

A hiperglicemia presente na obesidade, na resistência à insulina e do DM2 é responsável por altos níveis de stress oxidativo nestas situações, uma vez que causa bloqueio da cadeia transportadora de elétrons, gerando elevados níveis de espécies reativas

de nitrogênio e oxigênio (Nishikawa et al., 2000; Brownlee, 2005). Entretanto as razões pelas quais as desordens psiquiátricas tem prevalência aumentada na obesidade e podem ocorrer em conseqüência do DM2 ainda são desconhecidas.

1.5. OBJETIVOS

1.5.1. Objetivo geral

Em função das evidências atuais sobre o consumo de alimentos hiperpalatáveis e a obesidade por eles desencadeada, procuramos :

- avaliar os efeitos de ambos sobre o metabolismo intermediário, especificamente em relação aos fatores de risco para doenças cardiovasculares;
- verificar a ação destes alimentos e da obesidade gerada em aspectos comportamentais e parâmetros bioquímicos associados ao estresse oxidativo.

1.5.2. Objetivos específicos

- Avaliar a massa adiposa, tolerância à glicose, perfil lipídico, resistência à insulina, níveis de insulina, de adiponectina e de óxido nítrico em soro de ratos submetidos à dieta hiperpalatável;
- Avaliar a atividade das ectonucleotidases por meio da hidrólise de nucleotídeos da adenina;
- Avaliar o comportamento de ansiedade, locomoção, aprendizado e memória de ratos submetidos à dieta hiperpalatável;
- Investigar a existência de stress oxidativo em estruturas do sistema nervoso central de ratos submetidos à dieta hiperpalatável.

2 – ARTIGOS

2.1 – CAPÍTULO 1

EFFECTS OF A HIGHLY PALATABLE DIET ON LIPIDIC METABOLIC PARAMETERS, ADIPONECTIN LEVELS AND ADENINE NUCLEOTIDES HYDROLISYS

Em processo de finalização para ser submetido ao Journal of Nutritional Biochemistry

Effects of a highly palatable diet on lipidic metabolic parameters, adiponectin levels and adenine nucleotides hydrolisys.

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Abstract

Obesity reached epidemic proportion worlwide and is stimulated by the ready availability of food rich in fat and sugar (highly palatable diet), increasing the risks of obesity-associated pathologies. Adiponectin, an adipocitokine, is inversily proportional to body mass index and its serum levels are decreased in obesity and in cardiac patients, as the same way nitric oxide levels are decreased in these two situations. ATP, ADP,AMP and adenosine are molecules also linked to cardiovascular health and can be regulated by soluble and cell surface located ectonucleotidases. Alterations in the phisiologic hydrolysis of these nucleotides are related to cardiovascular complications. The aim of this study is evaluate the effects of a highly palatable diet, as a model of obesity induction, in metabolic parameteres, adiponectin levels and ectonucleotidases activity. Twenty male Wistar rats received two diferent diets during four months: standard chow (SC) and highly palatable (HP). Body weight, body fat mass, glucose intolerance, total cholesterol, HDL cholesterol, serum triacylglycerol (TAG), liver triacylglycerol and free glycerol were higher in HP group ($p<0.05$ and $p<0.01$), although insulin and adiponectin levels were not different between groups. However, nitric oxide had a trend to be lower in the HP group ($p=0.06$) and the hydrolysis of ATP, ADP and AMP were significantly lower in this same group ($p<0.05$ and $p<0.01$). In conclusion, the consumption of a highly palatable promotes subtle metabolic alterations involving cardiovascular system homeostasis and increasing the risk of cardiovascular diseases even when conventional diagnosis markers as insulin and adiponectin are not altered yet.

Key words : Highly Palatable Diet / Obesity / Adiponectin / Type 2 diabetes / Nucleotide Hydrolysis / Ectonucleotidases / Cardiovascular Disease

Introduction

Obesity can be considered the major syndrome of 21st century and is envolved in the etiology of several diseases as insulin resistance, hypertension, cardiovascular diseases and type 2 diabetes [1,2]. The most important cause of obesity is probably over-eating (coupled with inactivity) which is stimulated by the ready availability of food rich in fat and sugar [3]. The consumption of palatable foods (highly palatable diet) is capable to induce these metabolic alterations, being a decisive factor to increase adipose tissue as well as to developing of obesity-associated pathologies [4,5].

Adipose tissue secretes bioactive peptides, termed 'adipokines', which act locally and distally through autocrine, paracrine and endocrine effects [6]. Adiponectin, one of these citokines, is a 244 amino-acid protein synthesized and secreted exclusively by adipose tissue, displaying major insulin-sensitizing properties in skeletal muscle, liver and adipose tissue, antiatherogeinc effects and stimulates nitric oxide production in vascular endothelial cells, promoting vasodilatation [7,8]. Circulating adiponectin levels concentrations are inversely correlated with body fat mass and these levels are predominantly determined by visceral fat [9]. Obesity and insulin resistance negatively correlates with adiponectin concentration but decreased adiponectin was originarally described in patients with cardiovascular disease and for that reason changes in vascular system could be attributed to changes in circulating adiponectin concentration [8].

In vascular system extracellular adenine nucleotides ATP, ADP, AMP and its nucleoside adenosine have several biological roles [10]. Serum ATP can act as a vasolidatator or a vasoconstrictor, depending of its concentration in blood flow. Besides,

this nucleotide is substrate for ADP and AMP formation, which are platelet aggregants, and both can be hydrolised to adenosine, a pontent vasodilatator. In the extracellular space these nucleotides can be hydrolyzed by soluble and cell surface located ectonucleotidases.Thus, it has been postulated that the activity of these enzymes is essential for the maintenance of ATP/ADP/AMP/adenosine appropriate levels contributing to vascular homeostasis [11,12].

Exploring the effects of a highly palatable diet in metabolism and vascular system, the aim of this study is to verify the effects of this diet in metabolic parameters, adiponectin levels, ectonucleotidases activity and a possible conection between adiponectin levels and ectonucleotidases activity, acting together as regulators of vascular system.

Matherials and Methods

Animals and Diets

Twenty 60-day old male Wistar rats weighting from 210 to 230 *g* were obtained from the Central Animal House of UFGRS Biochemistry Department, Federal University of Rio Grande do Sul, Brazil. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.), at a room temperature of 22 $\pm$ 2°C. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Rats were divided into two groups: (1) the control group (SC, n=10), which received standard laboratory rat chow (65 % carbohydrate totally from starch, 25 % protein and 10% fat); and (2) the highly palatable diet group (HP, n=10), which received a enriched sucrose

diet (65 % carbohydrates being 34 % in condensed milk, 23% from starch and 8 % from sucrose, 25 % protein, 10 % fat). All animals had free access to food and water. After four months of diet, they were killed by decaptation and analyses were performed.

Glucose tolerance test

Glucose tolerance test was performed one week before the animals were sacrificed in each time. A 50 % glucose solution was injected into the animals (i.p, 2 mg/g) after 6h of starvation [13]. The blood was collected by a small puncture on tail immediately before and 30, 60, and 120 minutes after the injection. At each time, glucose was measured by a glucosimeter (AccuChek Active, Roche Diagnostics®, USA).

Blood and Tissue preparation

Animals were anesthetized by sodium tiopenthal (40 mg/Kg) and blood samples were collected by cardiac punction. Visceral and epididimal fat pads (which we will present as fat mass) were dissected and weighted. The blood was imediatelly collected and centrifuged at 5000 g by 10 minutes to obtain serum.

Metabolic parameters and nitric oxide determination

Serum metabolic parameters such as total cholesterol, LDL-cholesterol, HDL cholesterol and triacilglycerol (TAG) were measured by commercial kits (Roche Diagnostics, Indianapolis, USA). Serum glycerol was used as a marker of lipolysis by

indirect determination of free fatty acids and was measured with Free Glycerol Reagent Kit from Sigma Chemical Co. (St. Louis, MO, USA).

Hepatic TAG, a marker of insulin resistance, was measure using a 100 mg liver sample, homogenized in 1:20 saline solution (0.9%) and the assay was performed with an aliquot of this mixture, by commercial kits (Roche Diagnostics, Indianapolis, USA). Nitric oxide was determined with Greiss reagent, as described by Miranda et al., 2001 [14].

Measurement of ATP and ADP hydrolysis

ATP and ADP hydrolysis were evaluated using the method described by Oses et al.(2004) [15]. The reaction mixture containing 3.0mM ATP or ADP as substrate, 1.0-1.5 mg serum protein and 112.5 mM Tris-HCl, pH 8.0, was incubated at 37ºC for 40 minutes in a final volume of 2.0 mL. The reaction was stopped by the addition of 0.2 mL of 10% TCA. All samples were centrifiged at 5000 g for 5 min and the supernatant was used for measuring the amount of inorganic phosphate (Pi) released through a colorimetric assay [16]. Incubation time and protein concentrations were chosen to ensure the linearity of the reaction (results not shown). In order to correct non-enzymatic hydrolisys, we performed controls by adding serum after TCA. All samples were assayed in duplicate. Enzyme activities were expressed as nmol of Pi released per minute per miligram of protein.

Measurement of AMP hydrolysis

To evaluate the AMP hydrolysis we used a reaction mixture containing 3.0 mM AMP in 100 mM Tris-HCl, pH 7.5, incubated with 1.0-1.5 mg of serum protein at 37ºC in a

final volume of 0.2 mL. All other procedures were the same as described above of ATP and ADP hydrolysis.

Measurement of insulin and adiponectin levels

Serum insulin levels were measured by RIA (BioChem ImmunoSystems, Roma, Italy) and adiponectin was measure by Elisa kit (Linco Research, St Charles, MO, USA).

Protein determination

Protein was measured by the Comassie Blue method using bovine albumin as standard [17].

Statistical analysis

Data were analyzed using SPSS 10.0 software, with Student's T test for independent samples to parametric variables. Results are expressed as mean ± standard deviation.

Results

At the end of treatment, final body weight was different between SC and HP groups ($p<0.01$, Figure 1A) and the same is observed in the fat mass ($p<0.01$, Figure 1B), which were almost two fold higher in HP group. As we can observe in figure 1A and 1B, the gain of body weight related to fat mass was higher in HP group than in SC group (fat mass to body weight ratio : 0.04 in SC group ; 0.06 in HP group).

The glucose tolerance was lower in HP group and the higher glucose levels were found at 30 and 60 minutes after the injection ($p<0.01$, Figure 1C), compared to SC group.The glucose tolerance test was different between groups starting from 30 minutes after the glucose injection until the end of the test (120 minutes after), once HP group glucose levels remain statistically different from fasting glucose levels while SC group was not different ($p<0.05$, Figure 1C).

Both total cholesterol and HDL cholesterol of HP group were different between groups, being higher in HP group ($p<0.05$, Table 1). The serum TAG levels were two fold higher in HP group ($p<0.05$, Table 1) and the same was observed in the liver TAG content of this group ($p<0.01$, table1) and the free glycerol ($p<0.01$, Table 1). Altough glucose tolerance was altered in HP group, serum insulin and adiponectin levels were not different between the groups (Table 1). Nitric oxide was not statistically different between groups at four months of diet, although there is a trend to be higher in SC group ($p=0.07$, Figure 2A).

The hydrolisys of adenine nucleotides was significantly lower in HP group. In HP group ATP hydrolisys was 33% lower ($p<0.01$, figure 2B), ADP hydrolysis was 49% lower ($p<0.01$, figure 2C) and AMP hydrolysis was 23 % lower ($p<0.05$, figure 2D) when compared to SC group .

Discussion

The consumption of a highly palatable diet enriched with sucrose for four months was able to alter body composition, promoting gain of weight and fat more vigorously than a standard diet. As the same way, total cholesterol, HDL cholesterol, serum TAG, hepatic TAG and free glycerol reached elevated levels on the animals of HP group.

The sucrose consumption also caused severe glucose intolerance, once animals submitted to the diet had higher glucose plasmatic levels than control group, even 120 minutes after the i.p. glucose injection, which leads to type 2 diabetes instalation, even without changes in insulin and adiponectin levels between groups until this point. Associated to that, adenine nucleotides hydrolisys was decreased in HP group, since the diet considerably lowered ectonucleotidases activity. The trend to decrease nitric oxide levels in this same group corroborates with the diet's effect on ectonucleotidases activity.

Obesity, glucose intolerance and dyslipidemia are the most commom features of metabolic syndrome, a complex of alterations that frequently leads to type 2 diabetes and/or cardiovascular diseases [18,19] and is well know that consumption of diets enriched with fat, sucrose or both is positively associated to the development of this syndrome [20,3], although the most used models to induction of obesity and type 2 diabetes are high fat diets. The increase of body fat mass, caused by these diets, also leads to alterations in adipose tissue functions, including dysregulation of fatty acids metabolism, insulin resistance and changes in adipocitokines secretion [21,22]. In our study, we used high amounts of sugar, changing standard carbohydrate composition, without changes in dietary fat content. Animals feeded with the highly palatable diet developed alterations on body composition, glucose tolerance, lipid profile and fatty acids utilization, as the same way that occurs in high fat diets, but without differences in insulin and adiponectin levels. However, elevated hepatic TAG and free glycerol are indirect markers of insulin resistance, once these elevations occur by impaired ability of insulin to suppress endogenous glucose production and fatty acid oxidation on liver and defect in insulin suppression of lipolysis at

the level of adipocyte [23,24]. Therefore, insulin resistance already exists even without changes in serum insulin levels, as we can observe in our results.

Besides alterations in fatty acids metabolism and insulin actions, the increase of body fat mass are related with changes in adiponectin secretion [25,26]. Adiponectin, an adipocitokine, is the most abundant gene product in adipose tissue and exerts pro-insulin actions in muscle and liver, antiaterogenic and antiinflamatory functions in vascular endotelium and stimulate nitric oxide production and secretion [7,27,28]. Although obesity decreases serum adiponectin levels, we could not observed that in the animals of HP group, even they became obese. Our data corroborates with Barnea et al. (2006)[29] which also did not find alterations in serum adiponectin levels in a model of diet-induced obesity and insulin resistance in mice, although adiponectin and adiponectin receptors mRNA was decreased in muscle and liver. However, this work showed in the same animals that they had insulin sensitivity accompanied by impaired activity of adiponectin-related enzymes. For this reason, we propose with our results that metabolic alterations maybe starts before adiponectin levels decreases, as the same way occured with insulin parameters. We can reinforce that with the lower nitric oxide levels in HP group, one of the most expressive endogenous vasodilatator, which should follow adiponectin levels, once is stimulated by it, and that did not occur.

The lower activity of ectonucleotidades is another evidence that changes in metabolism may starts earlier than the most known alterations, based in the decreased hydrolysis of adenine nucleotides in the HP group. On vascular system, ATP and their metabolites (includind ADP, AMP and adenosine) are involved in the control of several biologic processes and these molecules function on blood vessels, as vasoconstrictors or

vasodilatators, is well know [10]. The ectonucleotidases exerts an essential role in blood flow regulation and in trombogenesis, by converting ATP, ADP and AMP, which stimulate vasoconstriction and platelets agregation, in adenosine, which exert the opposite functions [11,12]. Therefore, the homeostasis of ectonucletidases activity is important to avoid vascular trombosis and atherogenic process through formation and maintenance of adenosine levels [30,31].

Atherosclerosis is the cause of death of 80% of type 2 diabetic individuals as a consequence of untreated hyperglicemia and its alterations in adipose tissue, inflamatory response and vascular homeostasis [32,18]. Here we demonstrated that consumption of a highly palatable diet leads to body fat mass increase, metabolic alterations and type 2 diabetes in association with lower ectonucleotidases activity, promoting an appropriate environment to development of cardiovascular diseases, without changes in insulin and adiponectin levels. We concluded that possibly cardiovascular disease, type 2 diabetes and obesity complications have more subtle indicators and starts before most known markers are altered, as conventional diagnosis.

Acknowledgments

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Legends and figures

Figure 1. Characterization of the obesity model : body mass and glucose tolerance test after four months of a highly palatable diet intake. A) Final body weight (g); B) Final body fat mass (g); C) Glucose tolerance test : 0 (fasting), 30, 60 and 120 minutes after i.p. injection of a 50% glucose solution (2mg/g). SC= starndard chow; HP= highly palatable. * = $p<0.05$; **=$p<0.01$. Results are expressed as mean ± standard deviation.

Table 1. Metabolic parameteres of groups after four months of diets consumption. Results are expressed as mean ± standard deviation.

Figure 2. Measure of NO levels and ectonucleotidases activity in rat serum after four months of diets consumption. A) Serum NO levels; B) ATP hydrolysis; C) ADP hydrolysis; D) AMP hydrolysis. B,C, D = ectonucleotidases specific activity (nmol phosphate/min/protein mg). SC= standard chow; HP= highly palatable. * = $p<0.05$; **=$p<0.01$. Results are expressed as mean ± standard deviation.

Figure 1

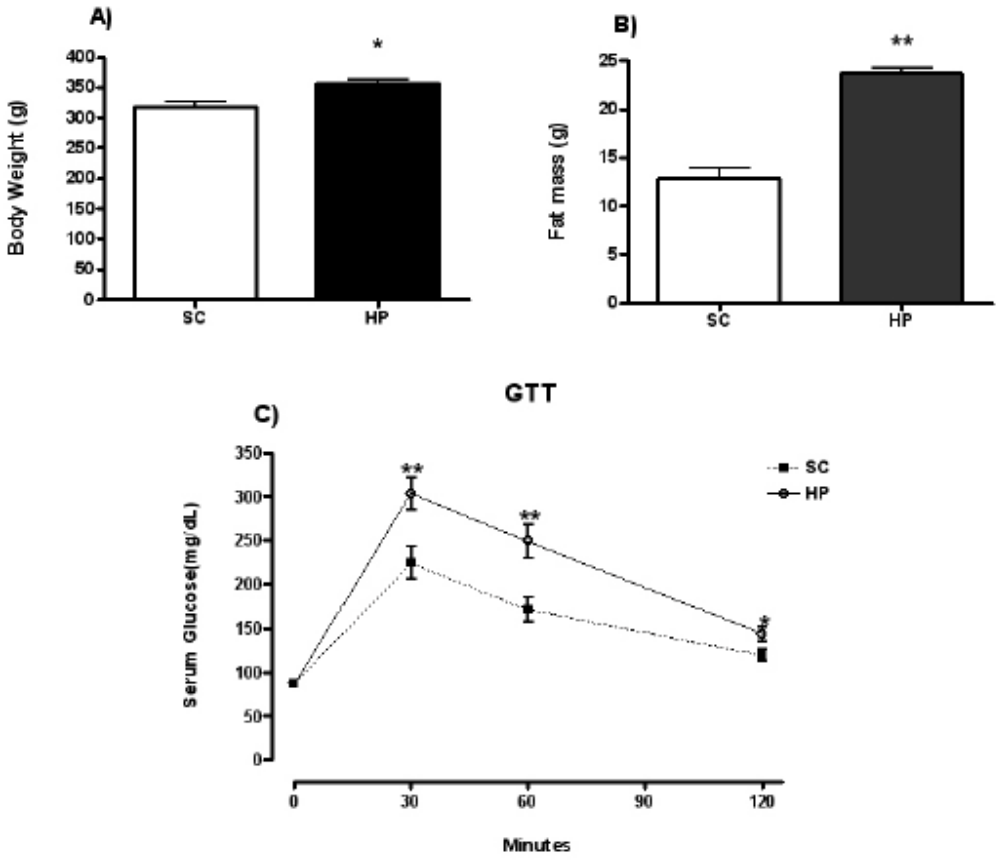
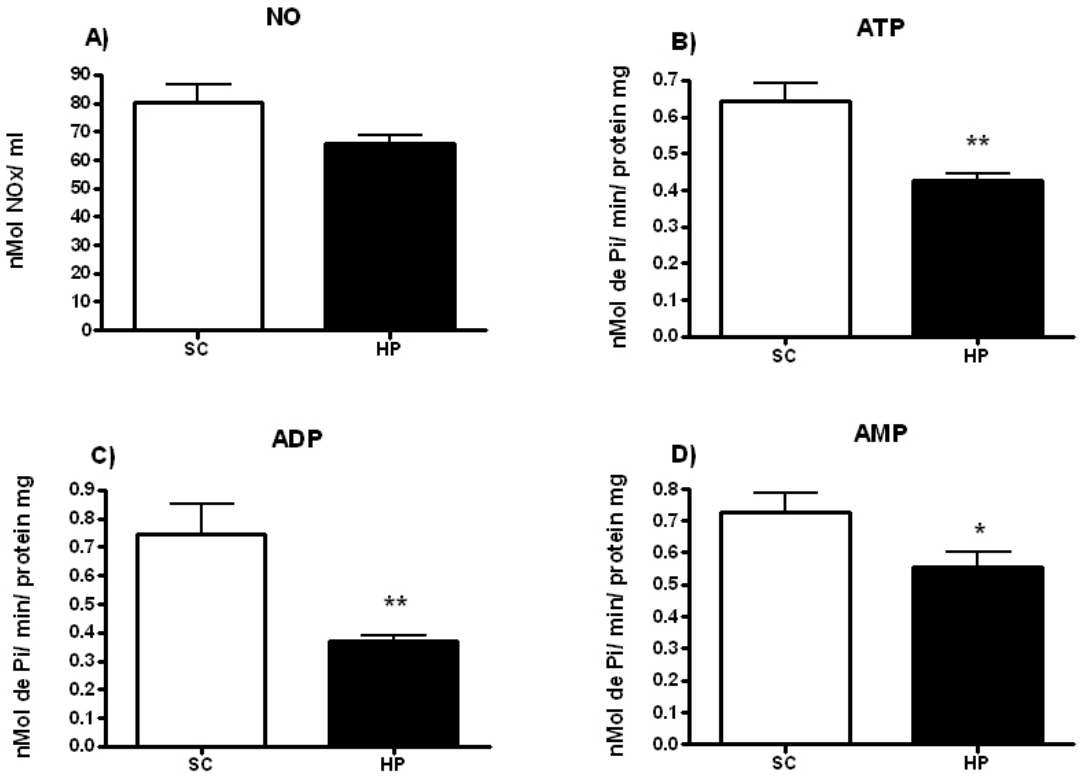

A)
Body Weight (g)
400
350
300
250
200
150
100
50
0
*
SC
HP
B)
Fat mass (g)
25
20
15
10
5
0
**
SC
HP
GTT
C)
Serum Glucose(mg/dL)
350
300
250
200
150
100
50
0
**
**
**
SC
HP
0
30
60
90
120
Minutes

Table 1 - Metabolic parameteres of groups after four months of diets consumption.

Groups	SC (n =10)	HP (n=10)
Total cholesterol (mg/dl)	47.0 ± 9.0	52.0 ± 7.0*
HDL cholesterol (mg/dl)	29.0 ± 5.0	34.0 ± 5.0*
Serum TAG (mg/dl)	67.0 ± 14.0	128.0 ± 70.0*
Liver TAG (mg%)	1.0 ± 0.3	2.0 ±0.3**
Free Glycerol (mg/ml)	0.01 ± 0.002	0.02 ± 0.003**
Insulin (µUI/ml)	85.0 ± 31.0	80.0 ± 26.0
Adiponectin (ng/ml)	43.0 ± 14.0	39.0 ± 7.0

SC= starndard chow; HP= highly palatable. * = $p<0.05$; **=$p<0.01$.

Figure 2


A)
NO
nMol NOx/ ml
90
80
70
60
50
40
30
20
10
0
SC
HP
B)
ATP
nMol de Pi/ min/ protein mg
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
SC
HP
C)
ADP
nMol de Pi/ min/ protein mg
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
SC
HP
D)
AMP
nMol de Pi/ min/ protein mg
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
SC
HP

2.2 – CAPÍTULO 2

HIGHLY PALATABLE DIET CONSUMPTION INCREASES PROTEIN OXIDATION IN RAT FRONTAL CORTEX AND ANXIETY LIKE BEHAVIOR

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Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety like behavior

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Abstract

Obesity is frequently associated with consumption of high amounts of sugar and/or fat. Studies have demonstrated a high prevalence of overweigh and obesity associated or not with increase rates of psychiatry disorders; in particular mood and anxiety disorders. Recent works have demonstrated an association between specific genes involved in oxidative stress metabolism and anxiety-like behavior. The aim of this study was to investigate the effect of a highly palatable diet enriched with sucrose in body fat mass composition, anxiety behavior and brain oxidative status. Twenty male wistar rats received two diferent diets during four months: standard chow (SC) and highly palatable (HP). Metabolic parameters, behavioral tests and oxidative stress status were evaluated. Body fat mass, insulin sensitivity and glucose tolerance was altered in the HP group ($p<0.01$). The same group spends less time in light compartment and had a lower risk assessment behavior ($p<0.05$) but no differences was observed in the open field test habituation ($p>0.05$). Protein degradation, DCF and TBARS levels was not different in the hippocampus between groups however there was higher levels of protein degration in frontal cortex of HP groups ($p<0.05$), although DCF and TBARS levels don't differ from SC group ($p>0.05$). In conclusion, our data suggest that the consumption of HP diet leads to an obese phenotype, increases protein oxidation in frontal cortex and appears to induce anxiety like behavior in rats.

Key words: obesity / highly palatable diet / anxiety / oxidative stress / frontal cortex

Introduction

In modern societies the consumption of high fat and high sugar diets as well as a sedentary lifestyle has been associated with metabolic derangements and impairment of brain function (Schrawen et al., 2000; Unger et al., 2001; Molteni et al., 2002, 2004; McGarry, 2002). Accordingly, in human studies overweigh and obesity maybe associated with increase rates of psychiatry disorders; in particular mood and anxiety disorders (Chakravarthy et al., 2004, Simon GE, 2006; Becker et al., 2001; Teegarden and Bale, 2007). Also, experimental studies have focusing on the molecular and behavioral response underlying the specific effects of high fat and sugar diet on neural cells (Molteni et al, 2002, 2004).

Colantuani et al., (2002) demonstrated that continuous excessive sugar consumption leads to an addiction profile and increases anxiety behavior when rats are deprivated from sugar, whereas when animals are refeeding they overeating. In addition to this altered behavior induced by excessive sugar intake, elevated level of plasmatic glucose has been considered the main source of free radicals production in glucose intolerant individuals (Brownlee, 2005).

The imbalance between high cellular levels of reactive oxygen species (ROS) in relation to cellular antioxidant defenses, namely oxidative stress, may be involved in the pathogenesis of several brain diseases. If not effectively removed ROS may cause oxidative cell injury (Gutteridge and Halliwell, 2000; Halliwell and Gutteridge, 2000). Protein damage, lipid peroxidation and energy failure caused by oxidative stress are frequently reported alterations that could affect neurons and consequently the brain functioning (Guix

et al., 2005; Moncada and Bolanos, 2006; Calabrese et al., 2006). Recently, a new insight emerged from the contradictory works of Hovatta et al. (2005) and Berry et al., (2007) in which they suggests a linking between different genes involved in oxidative stress metabolism and anxiety like behavior.

Up to our knowledge, the association between the consumption of a highly palatable diet sucrose enriched (HP), anxiety and brain oxidative damage has not yet been studied. Thus, the aim of this study was to investigate the effect of a HP diet in anxiety behavior and brain oxidative status.

Material and Methods

Chemicals

All reagents were of analytical grade. Thiobarbituric acid was obtained from Merck (Rio de Janeiro, Brazil), while 2'-7'-dichlorofluorescein diacetate, sodium dodecylsulfate, trichloroacetic acid and phenyl methyl sulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and Diets

Twenty 60-day old male Wistar rats weighting from 210 to 220 *g* were obtained from the Central Animal House of our Department. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.), at a room temperature of 22 $\pm$ 2°C. Animal care followed the official governmental guidelines in compliance with the

Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Rats were divided into two groups: (1) the control group (SC, n=10), which received standard laboratory rat chow (50 % carbohydrate, from starch, 22 % protein and 4 % fat); and (2) the highly palatable diet group (HP, n=10), which received a enriched sucrose diet (65 % carbohydrates being 34 % in condensed milk and 8 % from sucrose), 25 % protein, 10 % fat). All animals had free access to food and water.

Behavioral tests

After four months animals were allowed to explore an open field (two 5-min sessions on successive days) and a light-dark arena (one 5-min session). Behavioral testing was carried out in a special room with constant temperature (21 $\pm$ 2º C) and light conditions (60-W light), except for the light-dark exploration task (see details below). Before the sessions, animals were allowed to adapt to the experimental room for at least 1 h. All tasks were performed between 09:30 to 12:00 AM.

Open-field exploration

In two successive days, the rats were gently placed in the corner of a 40 x 50 x 60-cm box, the floor of which was divided into 3 x 4 cm squares, and left free to explore it for 5 min. The number of crossings from one square to another, groomings and rearings was counted. Latency to leave the first square was also measured.

Light-dark exploration task

The light-dark exploration task consisted of a 40 x 50 x 60-cm box divided equally into two compartments that were connected by a small opening (10.0 cm x 7.5 cm). The light compartment was illuminated under a 60-W light. The dark one received only part of the room illumination (at 20 W). The floor of each compartment was divided into 3 x 2 cm squares. Rodents are nocturnal animals preferring darker areas, and the decrease in the exploratory activity in the light area is taken as a measure of anxiety. Animals were gently placed in the corner of the light compartment and left free to explore it for 5 min. The following parameters were recorded: (1) the frequency of crossings and rearings from one square to another within the light or in the dark compartment; (2) the number of entries into the light compartment, defined as placing the four paws into this compartment; and (3) the total time spent in the light compartment. In addition, the risk assessment behavior index (RA, i.e. the number of investigations of the light compartment by placing some but not all paws) was recorded. After each trial, the apparatus was cleaned with an ethanol solution (70 %).

Glucose tolerance test

Glucose tolerance test was performed two days before the animals were sacrificed. A 50 %-glucose solution was injected into the animals (i.p, 2 mg/kg) after 6h of starvation. The blood was collected by a small puncture on tail immediately before and 30, 60, and 120 minutes after the injection. At each time, glucose was measured by a glucosimeter (AccuChek Active, Roche Diagnostics®, USA).

Tissue preparation and oxidative stress damage evaluation

One week after the behavioral tests, the rats were killed by decapitation. Visceral and epididimal fat pads were dissected and weighted. Cerebral frontal cortices and hippocampi were dissected out and immediately stored at -70°C until biochemical measurements, when they were homogenized in 10 volumes of ice-cold phosphate buffer (0.1 M, pH 7.4) containing 140 mM of KCl, 1 mM of ethylenediaminetetraacetic acid, and 1 mM of phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 960 g for 10 min and the supernatant was used for all subsequent analysis. All steps were carried out at 4°C.

In order to verify a possible impact of obesity and glucose levels in brain oxidative stress, we evaluated oxidative damage to proteins and lipids in the hippocampus and frontal cortex. The levels of oxidized fluorescent derivative (DCF), thiobarbituric acid-reactive substances (TBARS) and the protein residues of tyrosine and tryptophan were quantified in these structures, with all data expressed as percentages of the control group.

Free radical levels

An aliquot of the sample was incubated with 2'- 7'- dichlorofluorescein diacetate (100 μM) at 37°C for 30 min. The formation of DCF was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-2000; Hitachi, Tokyo, Japan). The formation of reactive oxygen species was quantified by using a DCF standard curve and results were expressed as picomoles of DCF formed per milligram of protein (Sriram et al., 1997).

Lipid peroxidation assay (TBARS)

Sample aliquots were incubated with 10 % trichloroacetic acid and 0.67 % thiobarbituric acid. The mixture was heated on a boiling water bath for 30 min, an equal volume of *n*-butanol was added, and the final mixture was centrifuged. The organic phase was collected for fluorescence measurements at excitation and emission wavelengths of 515 and 553 nm, respectively (Bromont et al., 1989). We used 1,1,3,3-tetramethoxypropane as the standard. TBARS levels are expressed as picomoles of malondialdehyde per milligram of protein.

Degradation of protein tyrosine residues and tryptophan residues

Sodium dodecylsulfate was added to sample aliquots (final concentration 0.1%). The tyrosine residues within solubilized proteins were determined fluorometrically at excitation and emission wavelengths of 277 and 320 nm, respectively (Gusow et al., 2002).

Sodium dodecylsulfate was added to sample aliquots (final concentration 0.1%). Tryptophan residues within solubilized proteins were determined fluorometrically at excitation and emission wavelengths of 280 and 345 nm, respectively (Bondy, 1996).

The determination of tyrosine and tryptophan residues is used as a tool for evaluates oxidative stress in proteins.

Protein assay

Total protein concentration was determined according to the method described by Lowry (1951) with bovine serum albumin as the standard.

Statistical analysis

Data were analyzed using SPSS 10.0 software. Body weight, glucose tolerance and open field habituation test results were analyzed by repeated-measures ANOVA and post-hoc Tukey's test. The Student's *t* test was used for body fat mass, light dark test and analyses of oxidative stress parameters. The level of statistical significance was set at 95 ($p < 0.05$). Results are expressed as mean ± standard error (S.E).

Results

Body Composition and Glucose Tolerance Test

There was no difference between groups in body weight ($p>0.05$) [$F(1.18) = 2.79$; $p = 0.112$]. However, visceral and epididimal fat mass in the HP group was higher than in the SC group (9 ± 3 vs. 5±1 and 7 ± 3 vs. 4 ± 1, respectively; $p < 0.001$), indicating that the HP diet increases the gain of fat mass (Table 1).

Additionally, after four months HP diet triggered changes in the glucose homeostasis, which is supported by an impaired glucose tolerance ($p<0.001$)[$F(3,54) = 8.80$; $p=0.0000$] (Figure 1A) and also by increased liver triacylglycerol (TAG) concentration (HP, 1.9 ± 0.3 vs SC, 0.9 ± 0.3 mg%; $p<0.01$, Table 1), an indirect parameter for demonstrate overall insulin resistance (Burget et al., 2006).

Behavioral Parameters

In the open field habituation test, there was no difference between groups in the number of crossings, rearings, and in the latency for leaving the first square ($p>0.05$)[$F(1,18)= 0.35$, 0.18, and 0.20, respectively, $p = 0.56$] (Fig. 1B). In the light-dark task, the HP group spent less time in the light compartment than the SC group ($p<0.05$, Fig. 1C) and the locomotion of this group was lower in the same compartment ($p<0.05$, Fig. 1D). The HP group also had a trend to decrease the locomotion behavior on dark compartment ($p= 0.07$; Fig. 1E) and higher risk assessment behavior index than controls ($p < 0.01$; Fig.1F).

Oxidative stress parameters

There was no difference between groups in hippocampus regarding the content of tyrosine and tryptophan, DCF levels, and TBARS production (data not shown).

A significant lower content of tyrosine and tryptophan was observed in HP compared to control group indicating the presence of high protein damage induced by diet ($p<0.05$, Fig. 2A). There was no difference between groups in DCF and TBARS levels ($p>0.05$, Fig.2B and Fig. 2C, respectively).

Discussion

The results presented here show that animals submitted to highly palatable diet enriched wit sucrose (HP) had glucose intolerance, increased in insulin resistance and fat body composition, despite no differences on total body weight. Taking together these findings suggest that HP rats have obese like phenotype and impairment in glucose metabolism. Furthermore, HP diet increased anxiety like behavior and oxidative damage in proteins of the frontal cortex.

In the dark-light choice task, animals submitted to HP diet spent less time in the light compartment and, at the same time, they showed a higher number of refusals for entering the light compartment, which according to Gingrich (2005) and Hovatta et al., (2005) is a well-accepted feature of anxious behavior. Open field task was used to evaluate the locomotion capacity of animals, plus anxiety like behavior as a complementary measure of the light dark task. The anxiety like-behavior appears to be unrelated to any gross motor alteration, since the locomotion in the open field task was normal.

Considerable evidence has been accumulated to demonstrate that feeding behavior is closely related to emotions. Many obese patients tend to eat more when they are emotionally tense or depressed or simply bored (Vaswani et al.,1983). Additionally, obese patients frequently have psychiatric co-morbidities. Although depressive symptoms are frequently attributed to obese patients, the associations between anxiety disorders and obesity often go unrecognized. In an obese population of UK, Fifty-six percent of patients met the minimum criteria for an anxiety disorder and forty-eight percent met the minimum criteria for depression (Tuthill et al., 2006).

Chronic elevation of glucose levels is one metabolic alteration triggered by this diet consumption is reported as the main source of free radical production in glucose intolerance situations. An overload of glucose blocks the flux of electrons transport chain, generating elevated levels of reactive nitrogen and oxygen species (Nishikawa et al., 2000; Brownlee, 2005). Recent data from several reports indicate that these free radicals are involved in the biochemical mechanisms underlying neuropsychiatric disorders in human (Ozcan et al., 2004). The free radicals usually affect biomolecules, such as, proteins, lipids and DNA, and mitochondria (Griffiths, 2000; Murray et al., 2003).

The HP group had decreased tyrosine and tryptophan residues in frontal cortex proteins, an important brain structure involved in behavioral regulation, and together with hippocampus, amygdala and hypothalamus, which are limbic regions, form part of several well-defined anxiety and fear-related circuits in the forebrain (Singewald et al., 2003; Hovatta et al., 2005). The formation of reactive nitrogen and oxygen species can alter protein conformation through reactions with aminoacids residues, being tyrosine and tryptophan some of the most susceptible aminoacids because they have lowest potentials reduction (Alvarez and Radi, 2003). Oxidation of proteins is a very common consequence of oxidative stress, leading to changes in structure and function of these molecules (Alvarez et al., 1999).

It has long been established that genetic contributions increase the vulnerability to anxiety disorders, but the precise genes involved are unknown. In a recent work, Hovatta et al., (2005) proposes that, in mouse brain, overexpression of genes related to antioxidant enzymatic defenses (glyoxalase 1 and glutathione reductase 1) increases anxiety behavior and that these genes have a causal role in the genesis of anxiety. Conversely, Berry et al (2007) showed that deletion of gene $p66^{Shc}$, which regulates reactive oxygen species metabolism and apoptosis, reduced pain sensitivity and anxiety, which is in line with our findings regarding ROS and anxiety. It is possible that genetic variability may at least in part responsible for these inter-species and interindividual differences. Moreover, it has been demonstrated that a high palatable diet was able to influence gene expression of proteins involved in the mechanisms underlying neuroplasticity in hipocampus (Molteni et

al., 2002). Thus, the influence of high palatable diet on genes related to oxidative stress and anxiety like behavior seems to be an interesting field for future investigations.

Considering the limitations of our methodological approach, and the many brain systems involved in anxiety disorders this study was not able to establish a causal effect among HP diet and/or behavioral and biochemical parameters. Additional oxidative stress, molecular and behavioral parameters could be useful in order to search for more robust associations.

In conclusion, our data suggest that the consumption of high sucrose diet leads to an obese phenotype, increases protein oxidation in frontal cortex and appears to induce anxiety like behavior in rats.

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Legend for figures

Table 1. Body weight and fat mass. SC= Standard Chow; HP = Highly Palatable. * = p <0.01

Figure 1. Metabolic and behavioral parameters: A**)** Glucose tolerance test (GTT), * = p<0.05; **= p<0.01; B**)** Open field habituation test (OF), first and second days; C**)** Light dark task (LD): time in light compartment; D**)** Light dark task(LD): locomotion in light compartment; E**)** Light dark task(LD): locomotion in dark compartment; F**)** Light dark task: risk assessment behavioral index (RAB). SC= Standard Chow; HP= Highly Palatable. Results are expressed as mean ± standard error. * = p <0.05, ** = p<0.01.

Figure 2. Oxidative stress in the frontal cortex: A) Tyrosine and tryptophan residues levels (TIR/TRY); B) DCF levels; C) TBARS levels. SC= Standard Chow; HP= Highly Palatable. Results are expressed as mean ± standard error. * = p<0.05.

Table 1

Groups	SC (n =10)	HP (n=10)
Initial Body Weight (g)	219.0 ± 18.0	225.0 ± 18.0
Final Body Weight (g)	350.0 ± 35.0	375.0 ± 40.0
Visceral fat mass (g)	5.0 ± 1.0	9.0 ± 3.0*
Epididimal fat mass (g)	4.0 ± 1.0	7.0 ± 3.0*
Liver TAG (mg%)	0.9 ± 0.3	1.9 ± 0.3*

Figure 1

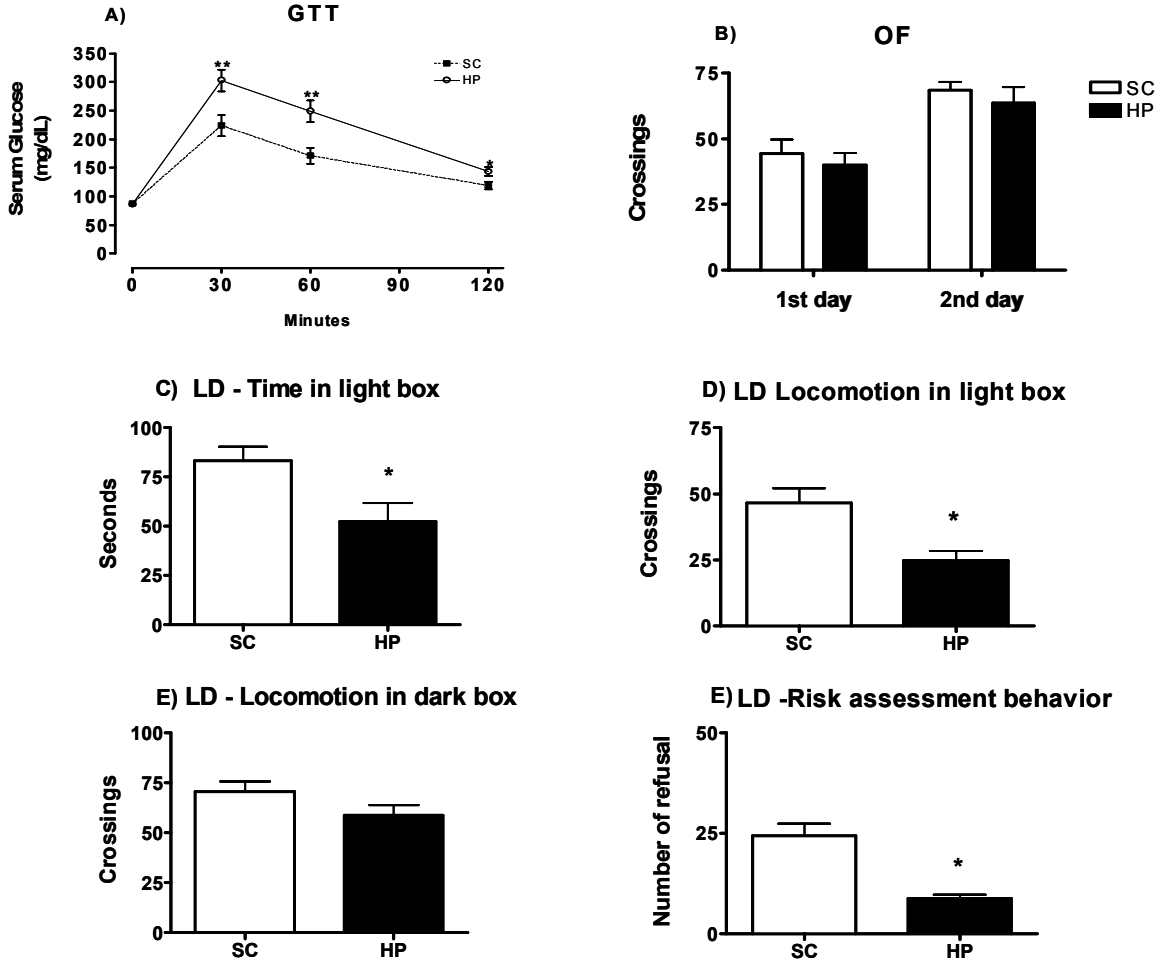
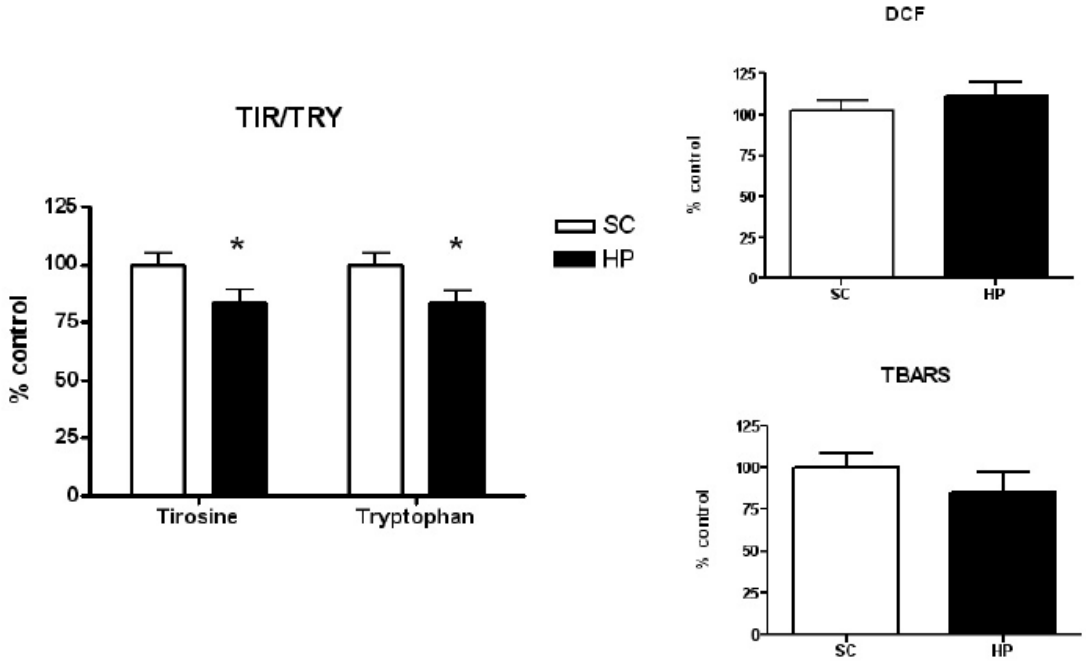

A)
GTT
Serum Glucose (mg/dL)
350
300
250
200
150
100
50
0
SC
HP
**
**
0
30
60
90
120
Minutes
B)
OF
Crossings
75
50
25
0
SC
HP
1st day
2nd day
C) LD - Time in light box
Seconds
100
75
50
25
0
*
SC
HP
D) LD Locomotion in light box
Crossings
75
50
25
0
*
SC
HP
E) LD - Locomotion in dark box
Crossings
100
75
50
25
0
SC
HP
E) LD -Risk assessment behavior
Number of refusal
50
25
0
*
SC
HP

Figure 2


TIR/TRY
% control
125
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Tirosine
Tryptophan
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HP
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SC
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TBARS
% control
125
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SC
HP

3. DISCUSSÃO

O aumento alarmante da obesidade nos últimos anos é atualmente um problema de saúde pública mundial. Muitas são as causas envolvidas em sua etiologia, desde alterações genéticas envolvidas com os genes das melanocortinas e da leptina até a inatividade física, porém o consumo exagerado de alimentos hiperpalatáveis e sua alta disponibilidade é, provavelmente, a maior causa dos níveis epidêmicos da obesidade (Cheng 2005, Erlanson-Albertsson, 2005).

O consumo de alimentos palatáveis estimula no SNC regiões límbicas relacionadas à sensação de prazer, euforia e recompensa, envolvendo ação de neurotransmissores e opióides, modulados pela própria alimentação (Colantuani et al., 2002). Esses estímulos afetam o comportamento alimentar de tal forma que o consumo a longo prazo deste tipo de alimento pode ser comparado com padrões de adição, devido ao feedback positivo gerado no sistema de recompensa, chamado "come back for more" (Kelley et al., 2002). A ingestão excessiva e contínua de açúcar já é descrita como um promotor deste padrão de adição (Colantuani et al., 2002).

O consumo regular de alimentos palatáveis promove obesidade e por conseqüência, hiperglicemia e resistência à insulina. Em nossos resultados observamos que os animais submetidos à dieta por 4 meses desenvolveram hiperglicemia severa, característica de diabetes tipo 2, acompanhada de aumento do glicerol sérico (um indicador indireto de ácidos graxos livres no sangue) e esteatose hepática (acúmulo de gordura no fígado). Essas alterações ocorrem por uma incapacidade da insulina de inibir a síntese endógena de glicose e a oxidação de ácidos graxos no fígado, bem como de inibir a lipólise à nível de

adipócito (Utzschneider and Kahn, 2006), embora os níveis séricos de insulina não estivessem alterados em nosso estudo. A sinalização insulínica diminuída ou inexistente também pode afetar o sistema cardiovascular, uma vez que este hormônio ativa a enzima óxido nítrico sintase endotelial (eNOS). O óxido nítrico é o mais potente vasodilatador endógeno e sua síntese ocorre pela via da fosforilação do susbstrato receptor de insulina1 (IRS1) → PI3K→ Akt, que ativa a eNOS. Esta mesma sinalização inibe a produção de moléculas vasoconstritoras, que geram uma resposta aumentada quando existe resistência insulínica, caracterizando uma disfunção endotelial (Yudkin et al., 2005). A disfunção endotelial é um marcador inicial da aterosclerose e muitos são os fatores de risco que predispõe a esta, podendo antes mesmo disso, causar a própria disfunção endotelial (Cheng, 2005).

Alterações nas citocinas secretadas pelo tecido adiposo também influenciam a funcionalidade do endotélio. A adiponectina é uma adipocitocina muito atuante no sistema cardiovascular por diminuir a adesão de monócitos, os fatores de coagulação e promover vasodilatação por meio de NO, além de sua ação pró-insulina. Não é à toa que pacientes cardíacos possuem menores níveis circulantes de adiponectina, que tem sua secreção e função também comprometidas em pacientes obesos, já que o aumento do tecido adiposo é negativamente correlacionado com os níveis desta adipocitocina (Cnop et al., 2003, Real 2004). Não conseguimos observar isto em nosso trabalho, pois embora os níveis de NO estivessem diminuídos, o mesmo não ocorreu com a adiponectina, que ainda não havia sofrido nenhuma alteração sérica. Por esta razão, levantamos a hipótese de que alterações mais sutis, porém já comprometedoras, possam já estar ocorrendo antes mesmo que

maiores marcadores e moduladores, como adiponectina e insulina séricas, estejam alterados. Corrobora com isto, a resistência à insulina existente no fígado e no tecido adiposo mesmo sem hipo ou hiperinsulinismo nos animais. A dislipidemia apresentada, que é um fator de risco clássico para doenças cardiovasculares, é mais uma alteração complementar de nossa hipótese.

O sistema cardiovascular é um dos mais afetados pela instalação do DM2 e de suas comorbidades associadas, uma vez que 80% das mortes de pacientes diabéticos ocorre por aterosclerose e três quartos destas são por doenças coronarianas (Cheng, 2005). Ainda neste sistema, o ATP extracelular e seus metabólitos (incluindo ADP, AMP e adenosina) estão envolvidos no controle de diversos processos biológicos. A importância destes nucleotídeos e seus derivados na corrente sangüínea atuando em processos de vasodilatação, vasoconstrição, agregação plaquetária, inflamação e dor é bem estabelecida (Frassetto et al., 1993; Ravelick e Burnstock, 2003). Sabe-se que o ATP liberado na corrente sangüínea, dependendo da sua concentração, pode atuar como um vasodilatador ou vasoconstritor. Além disso, é substrato para formação de ADP, um proagregante plaquetário, e ambos podem ser hidrolisados até adenosina, um potente vasodilatador. Os nucleotídeos extracelulares podem ser hidrolisados por várias nucleotidases, que estão localizadas em superfícies celulares e solúveis nos meios extracelulares, sendo o endotélio vascular um de seus principais sítios de localização e atuação. Estas enzimas exercem um papel importante na regulação do fluxo sangüíneo e da trombogênese. As nucleotidases convertem o ADP, um proagregante, em adenosina, uma molécula antiagregante, o que ajuda a controlar a agregação plaquetária intravascular (Kaczmarek et al., 1996; Zimmermann et al., 2001). Portanto, o controle das atividades dessas nucleotidases são importantes para impedir

processos de formação de trombos vasculares pela formação e manutenção do equilíbrio dos níveis da adenosina (Gayle et al., 1998). A diminuição da hidrólise destes nucleotídeos em nosso modelo de obesidade e DM2 é um fator importante a ser considerado para o desenvolvimento da aterosclerose, em concordância com o conjunto de alterações apresentadas, independente do decréscimo de insulina e adiponectina séricas. O perfil apresentado por estes animais sugere que possivelmente as doenças cardiovasculares, o DM2 e a própria obesidade tenham complicações que iniciem antes que alterações de diagnóstico convencional apareçam.

Paralelamente com esses efeitos no metabolismo intermediário, algumas mudanças nas funções cerebrais e cognitivas tem sido descritas neste mesmo modelo de obesidade. Estudos populacionais com indivíduos com sobrepeso e obesidade mostram uma possível maior incidência de desordens psiquiátricas nos mesmos, especialmente transtornos de humor e ansiedade (Chakravarhty et al., 2004, Simons, 2006, Teegarden and Bale, 2007). Em concordância com estas afirmações, observamos nos animais submetidos à dieta hiperpalatável, um comportamento mais aversivo e menos explorador, alterações estas, características de ansiedade aumentada em modelos animais (Gingrich,2005). Muitas evidências científicas mostram que o comportamento alimentar é fortemente ligado à emoções, indicando que uma grande maioria de indivíduos obesos alcançam critérios mínimos de ansiedade e depressão apresentando freqüentemente maiores comorbidades psiquiátricas do que pacientes não-obesos(Vaswani et al., 1983; Tuthill et al., 2006). Os mecanismos ligando estas duas patologias ainda não foram elucidados, porém o estresse pode ser um de seus fatores desencadeantes.

O estresse oxidativo está muito associado aos mecanismos bioquímicos de desordens psiquiátricas (Ozcan et al., 2004) e trabalhos recentes mostram que a diminuição da produção de RL é capaz de diminuir a ansiedade em modelos animais (Berry et al., 2007). A oxidação de proteínas, lipídios e até mesmo do DNA promovida por radicais livres (RL) ocasiona alteração da estrutura e perda da função destas moléculas e em nosso trabalho constatamos uma maior oxidação nas proteínas do córtex frontal dos animais submetidos à dieta, sendo que o córtex frontal é uma importante estrutura envolvida na regulação comportamental, fazendo parte de circuitos cerebrais relacionados à ansiedade e medo (Singewald et al., 2003; Hovatta et al., 2005). É importante salientar que este aumento de ansiedade e de proteínas oxidadas ocorreu em animais hiperglicêmicos e que a hiperglicemia crônica é a principal fonte de produção de RL em indivíduos intolerantes à glicose e diabéticos (Brownlee, 2005). Entretanto, considerando os muitos sistemas envolvidos nas desordens de ansiedade, não é possível ainda estabelecer um efeito causal entre esta alteração e os fatores associados citados.

Considerando todos os aspectos avaliados, concluímos que a obesidade induzida pelo consumo de uma dieta hiperpalatável altera parâmetros metabólicos promotores de um microambiente propício à doenças cardiovasculares, com alterações mais sutis do que as tradicionalmente investigadas, além de aumentar os níveis de ansiedade e o estresse oxidativo no SNC, promovendo também mudanças comportamentais, influenciadas pelo tipo de alimentação adquirida.

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