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Green tea extract improves high fat diet-induced hypothalamic inflammation, without affecting the serotoninergic system☆

Marcos H. Okuda*, Juliane C.S. Zemdegs, Aline A. de Santana, Aline B. Santamarina, Mayara F. Moreno, Ana C.L. Hachul, Bruno dos Santos, Claudia M. Oller do Nascimento, Eliane B. Ribeiro, Lila M. Oyama

Departmento de Fisiologia, Disciplina de Fisiologia da Nutrição, Universidade Federal de São Paulo, São Paulo, SP, Brazil

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Abstract

To investigate possible mechanisms of green tea's anti-obesity and anti-diabetic effects in the hypothalamus, the central regulator of metabolism, of mice fed with high-fat diet (HFD), we analyzed proteins of the toll-like receptor 4 (TLR4) pathway and serotoninergic proteins involved in energy homeostasis. Thirty-day-old male Swiss mice were fed with HFD rich in saturated fat and green tea extract (GTE) for 8 weeks. After that, body weight and mass of fat depots were evaluated. Oral glucose tolerance test was performed 3 days prior to euthanasia; serum glucose, insulin and adiponectin were measured in fasted mice. Hypothalamic TLR4 pathway proteins, serotonin receptors 1B and 2C and serotonin transporter were analyzed by Western blotting or enzyme-linked immunosorbent assay. A second set of animals was used to measure food intake in response to fluoxetine, a selective serotonin reuptake inhibitor. Mice fed with HFD had increased body weight and mass of fat depots, impaired oral glucose tolerance, elevated glucose and insulin and decreased adiponectin serum levels. TLR4, IκB-α, nuclear factor κB p50 and interleukin 6 were increased by HFD. Concomitant GTE treatment ameliorated these parameters. The serotoninergic system remained functional after HFD treatment despite a few alterations in protein content of serotonin receptors 1B and 2C and serotonin transporter. In summary, the GTE attenuated the deleterious effects of the HFD investigated in this study, partially due to reduced hypothalamic inflammation.
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Keywords: Green tea extract; High-fat diet; Hypothalamus; Neuroinflammation; Serotonin

1. Introduction

Obesity is an inflammatory disease, characterized by increased synthesis and secretion of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α), and decreased anti-inflammatory signals, including adiponectin [1]. One of the major causes of obesity is chronic positive energy balance, which varies according to extrinsic factors (e.g., food palatability and portion size) and intrinsic factors (e.g., satiety and hunger) [2].

The hypothalamus is the central regulator of energy homeostasis, and it receives nutritional, hormonal and neural information about the metabolic and nutritional status from the body [3]. It also regulates glucose homeostasis directly through the autonomic nervous system and indirectly by regulating insulin and glucagon secretion [4]. Dysfunction in these mechanisms may lead to weight gain and ultimately to obesity [5].

An important mechanism involved in hypothalamic dysfunction is neuroinflammation, which is observed in diet-induced obesity, and one of the key features is resistance to insulin [6] and leptin [7]. The toll-like receptor 4 (TLR4) pathway mediates the inflammatory process induced by diet rich in saturated fats, leading to pro-inflammatory cytokines synthesis and secretion [8], and inhibition of downstream signals of the TLR4 pathway — such as inhibitor of κB kinase β/nuclear factor κB (IKKβ/NF-κB) — in the hypothalamus prevented diet-induced obesity, as well as insulin and leptin resistance [9].

Prevention of obesity costs much less than its treatment and daily consumption of green tea (*Camellia sinensis*) is an interesting approach, as it induces weight loss or weight maintenance in adult subjects [10]. We demonstrated that green tea extract (GTE) reduced mass of adipose tissue depots and attenuated the mesenteric adipose tissue inflammatory response to high-fat diet (HFD) [11]. Moreover, other researchers demonstrated that polyphenols from green tea interacts with the TLR4 pathway [12,13].

One of the regulatory mechanisms of energy homeostasis in the hypothalamus involves the serotoninergic system. Its pharmacological stimulation induces anorexia in both humans and rodents [14,15],

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* Corresponding author. Universidade Federal de São Paulo Disciplina de Fisiologia da Nutrição, Rua Botucatu 862, Edifício de Ciências Biomédicas 2nd Floor, Postal Code 04023-062, São Paulo/SP, Brazil. Tel./fax: +55-11-55764765.

E-mail address: marcos.okuda@gmail.com (M.H. Okuda).

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whereas its inhibition increases food intake [16]. Moreover, serotonin signalling crosstalks with insulin and leptin signalling, two important metabolic factors that also regulate energy homeostasis [17].

The physiological effects of serotonin (5-hydroxytryptamine, 5-HT) are mediated by specific receptors, and the serotonin receptors 1B and 2C (5-HT1BR and 5-HT2CR, respectively) are involved in the control of food intake. Mice deficient in 5-HT1BR [18] or 5-HT2CR display hyperphagia [19], but only the latter develops obesity. Chronic consumption of HFD impairs the anorexigenic effects of serotonin by reduced post-prandial insulin and serotonin in extracellular regions of the hypothalamus [20] and altered turnover of serotonin [21].

We investigated the anti-inflammatory effects of GTE in the hypothalamus of HFD-fed mice. In addition, we analyzed the effects on the hypothalamic serotoninergic system, which contributes to energy homeostasis and may be altered by HFD.

2. Methods and materials

All procedures were approved by the ethics committee of Universidade Federal de São Paulo (protocol no. CEP 0883/11).

2.1. Experimental protocol

Three-week-old male Swiss mice were purchased from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia and given 1 week of acclimation in our facility, under controlled 12-h light–dark cycle (lights on at 6:00), temperature (24±1°C) and free access to standard chow food pellets and tap water. After that, mice were distributed semi-randomly into the following groups so that the mean initial body weight was similar among groups (within group weight variability of 4.3g, maximum): control diet+vehicle (control), control diet+GTE (control-GTE), high-fat diet+vehicle (HFD) and high-fat diet+GTE (HFD-GTE). As a daily routine (from 8:00 to 10:00), all mice were weighed in order to calculate the amount of GTE for each mouse (50mg/kg of body mass/day), then GTE was freshly prepared in tap water, and the GTE (0.1ml, different concentrations) or vehicle (0.1ml) was administered po by a single gavage, using a feeding needle. Treatments started when mice were 4 weeks old and carried on for a total period of 8 weeks.

The first treatment group (84 in total) was used for the oral glucose tolerance test (OGTT), performed 3 days before the end of the eighth week of treatment. We treated another group (42 in total) under the same conditions to evaluate food intake response to fluoxetine injection. Both procedures are described below.

2.2. Diets

The control diet (4% soybean oil) and HFD (4% soybean oil; 31.2% lard) were manufactured in-house (see Table 1 for diet composition). The GTE (TEAVIGO) was a kind gift from DSM Nutritional Products Ltd., Basel, Switzerland, and contained >90% of epigallocatechin-3-gallate (EGCG), 0.0% caffeine, ≤10ppm of heavy metals, ≤3ppm of arsenic and ≤1ppm of lead, as informed by the provider (product code 5009227, lot no. UQ00522013, analysis no. 01815600).

2.3. OGTT

The OGTT was performed 3 days before euthanasia, from 8:00 to 11:00. After an overnight fast, basal glycemia was measured from the tail vein using a glucometer (Accu-Check, Roche Diagnóstica Brasil Ltda., São Paulo, SP, Brazil). Immediately after the first measure, glucose (1.5g/kg) [22] was administered po, using with a feeding needle, and glycemia was measured again after 15, 30, 60, 90 and 120min to obtain the area under the glycemic curve.

Table 1
Composition of the control diet and HFD (modified AIN-93), growth (G) or maintenance (M)

	Control diet (G/M)	HFD (G/M)
Corn starch, %	62.95/72.07	40.92/40.87
Casein, %	20.0/14.0	13.95/14.0
Soya oil, %	7.0/4.0	7.0/4.0
Lard, %	–	28.08/31.2
Cellulose, %	5.0	5.0
Vitamin mix, %	1.0	1.0
Mineral mix, %	3.5	3.5
L-Cystine, %	0.3/0.18	0.3/0.18
Choline bitartrate, %	0.25	0.25
Butylhydroquinone, g/kg	0.014/0.008	0.014/0.008
Energy, kJ/g	16.5/15.9	22.4/22.4

2.4. Food intake in response to fluoxetine

To investigate the effects of HFD and GTE treatment on the serotoninergic system, we used fluoxetine, a selective serotonin reuptake inhibitor that induces hypophagia [23]. At the last week of treatment, mice were individualized and fasted for 6 h (12:00 to 18:00). Four days before euthanasia, at 17:30, intraperitoneal (ip) injection of saline (0.9%) or fluoxetine (10mg/kg of body weight) was performed. Thirty minutes later, food pellets were presented, and after 2 h, food intake was measured. The selected dose was based on studies by others [23,24], and we performed a pilot study with different doses of fluoxetine (1, 5 or 10mg/kg of body weight), and the hypophagic effect was only observed with the 10mg/kg dose. All mice served as their own control and were kept individualized until euthanasia.

2.5. Tissue sampling

To eliminate any stress caused by the OGTT or the fluoxetine test, euthanasia was carried out after 3 days of these procedures. During this interval, treatments were not interrupted.

At the end of the eighth week of treatment, mice (10–12h of overnight fast) were weighed and euthanized by decapitation. The hypothalami were dissected, frozen in liquid nitrogen and stored at −80°C for further analysis. Epididymal (E-WAT), retroperitoneal (R-WAT) and mesenteric (M-WAT) white adipose tissues (WAT) were dissected and weighed. Relative mass of WATs was calculated as % of total body weight and the sum of the three WAT pads ($\sum$-WAT) was also calculated.

2.6. Serum analysis

Trunk blood was collected, centrifuged to obtain serum and stored at −80°C for further analysis. The serum glucose, triacylglycerol, total cholesterol and high-density lipoprotein cholesterol concentrations were measured using a commercial enzymatic colorimetric kit (Labtest, Lagoa Santa, MG, Brazil). The insulin and adiponectin serum concentrations were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits (Merck Millipore, Billerica, MA, USA).

2.7. Protein extraction and Western blotting

The hypothalami were homogenized in protein extraction buffer (Tris–HCl, 0.1M, pH7.5; aprotinin, 0.1mg/ml; sodium pyrophosphate, 0.1M; sodium fluoride, 0.1M; EDTA, 0.01M; sodium orthovanadate, 0.01M; and PMSF, 0.002M), mixed with Triton X-100 (10%) and kept on ice for 30min and centrifuged (12,000g/40min/4°C). The supernatant was collected and protein content was analyzed with a Bradford assay kit (Bio-Rad, Hercules, CA, USA). Proteins were denatured at 100°C/5min in Laemmli buffer (bromophenol blue, 0.1%; sodium phosphate, 1M; glycerol, 50%; SDS, 10%; and DTT, 0.2M). Samples were loaded (25μg of total protein) and separated in SDS-PAGE gels (10%) in a Bio-Rad miniature slab gel apparatus. The electrotransfer of proteins from gels to nitrocellulose membranes was performed for ~1.5h/4 gels at 15V (constant) in a Bio-Rad semi-dry transfer apparatus, in transfer buffer containing methanol (20%) and SDS (0.02%). The membranes were rinsed thoroughly with wash buffer (Tris–HCl, 0.01M; NaCl, 0.15M; and Tween 20, 0.02%), blocked with bovine serum albumin (BSA) buffer (wash buffer with BSA, 1%) for 2 h and incubated with primary antibodies (1:1000) overnight at 4°C in blocking buffer. The membranes were washed 3 times for 10min and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 to 1:10,000) for 1 h at room temperature. The membranes were rinsed 3 times for 10min and chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) were visualized in a gel documentation system (Alliance 4.7, UVitec, Cambridge, UK). Band intensities were calculated with Scion Image (Scion Corporation 4.0.3.2) and standardized with β-tubulin.

The following primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA): TLR4 (sc-10741), MyD88 (sc-11356), TRAF6 (sc-7221), p-IKKα/β (sc-21661), total IKKβ (sc-34673), total IκB-α (sc-847), p-NF-κB p50 (sc-101744), total NF-κB p50 (sc-53744), p-NF-κB p65 (sc-101748), total NF-κB p65 (sc-71675), 5-HT1BR (sc-1460-P), 5-HT2CR (sc-10802) and serotonin transporter (sc-13997). Anti-β-tubulin (#2146) was purchased from Cell Signalling Technology (Danvers, MA, USA). Secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.8. ELISA

Hypothalamic homogenate was obtained exactly as described above, using the same protein extraction buffer. Quantitative assessment of TNF-α and IL-6 proteins was carried out using ELISA kit (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA) following the recommendations of the manufacturer. The results obtained were then standardized for total protein content.

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