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Hypothalamic mitochondrial abnormalities occur downstream of inflammation in diet-induced obesity

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ABSTRACT

Hypothalamic dysfunction is a common feature of experimental obesity. Studies have identified at least three mechanisms involved in the development of hypothalamic neuronal defects in diet-induced obesity: i, inflammation; ii, endoplasmic reticulum stress; and iii, mitochondrial abnormalities. However, which of these mechanisms is activated earliest in response to the consumption of large portions of dietary fats is currently unknown. Here, we used immunoblot, real-time PCR, mitochondrial respiration assays and transmission electron microscopy to evaluate markers of inflammation, endoplasmic reticulum stress and mitochondrial abnormalities in the hypothalamus of Swiss mice fed a high-fat diet for up to seven days. In the present study we show that the expression of the inflammatory chemokine fractalkine was the earliest event detected. Its hypothalamic expression increased as early as 3 h after the introduction of a high-fat diet and was followed by the increase of cytokines. GPR78, an endoplasmic reticulum chaperone, was increased 6 h after the introduction of a high-fat diet, however the actual triggering of endoplasmic reticulum stress was only detected three days later, when IRE-1α was increased. Mitofusin-2, a protein involved in mitochondrial fusion and tethering of mitochondria to the endoplasmic reticulum, underwent a transient reduction 24 h after the introduction of a high-fat diet and then increased after seven days. There were no changes in hypothalamic mitochondrial respiration during the experimental period, however there were reductions in mitochondria/endoplasmic reticulum contact sites, beginning three days after the introduction of a high-fat diet. The inhibition of TNF-α with infliximab resulted in the normalization of mitofusin-2 levels 24 h after the introduction of the diet. Thus, inflammation is the earliest mechanism activated in the hypothalamus after the introduction of a high-fat diet and may play a mechanistic role in the development of mitochondrial abnormalities in diet-induced obesity.

1. Introduction

Hypothalamic dysfunction has been widely studied in experimental obesity models [Reviewed in (Velloso and Schwartz, 2011,Araujo et al., 2016,Valdearcos et al., 2015,Cai, 2013,Kalin et al., 2015)]. Dietary consumption of large amounts of fats can damage specific hypothalamic neuronal subpopulations, leading to an imbalance between caloric intake and energy expenditure and resulting in anabolism (Velloso and Schwartz, 2011,Araujo et al., 2016,Valdearcos et al., 2015,Cai, 2013,Kalin et al., 2015,Cavadas et al., 2016). Studies have identified three distinct mechanisms involved in the pathophysiology of the hypothalamic damage in obesity: inflammation (De Souza, Araujo, Bordin et al., 2005,Zhang et al., 2008,Milanski et al., 2009,Thaler et al., 2012), endoplasmic reticulum stress (ERS) (Zhang et al., 2008,Milanski et al., 2009,Ozcan et al., 2009) and mitochondrial dysfunction (Schneeberger et al., 2013,Dietrich et al., 2013). Saturated fats present in the diet can trigger inflammation by activating microglia TLR4 signaling and ERS, which induce the expression of chemokines, such as fractalkine and MCP1, and cytokines, such as TNFa, IL1b and IL6 that act upon key neurons of the hypothalamus leading to leptin and insulin resistance and, eventually, to neuronal apoptosis. In addition, studies have shown that dietary fats can also disturb mitochondria dynamics in hypothalamic neurons and this

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impacts directly in the regulation of whole body energy homeostasis. Despite the extensive work performed in order to characterize the mechanisms leading to hypothalamic abnormalities in obesity, it is currently unknown, which of these mechanisms is triggered earliest.

In the present study, we evaluated key elements involved in the early steps of activation of inflammation, ERS and mitochondrial dysfunction in the hypothalamus of mice fed a high-fat diet (HFD) for 0–7 days. We show that the chemokine fractalkine (CX3CL1) is the earliest marker undergoing modulation by the diet. This was followed by activation of ERS and finally, at a later stage, by changes in mitochondrial dynamics markers.

2. Methods

2.1. Experimental animals

Swiss mice were purchased from the University of Campinas Breeding Centre and were maintained in a 12 h light/dark cycle at 22–24 °C. The access to diet and water was *ad libitum*. Chow (control diet) consisted of 10% kcal from fat, 20% kcal from protein and 70% kcal from carbohydrates. The high-fat diet (HFD) consisted of 60% kcal from fat, 15% kcal from protein and 25% kcal from carbohydrates [the complete composition of the HFD has been published elsewhere (Cintra et al., 2012)]. In all experiments, six-week-old male mice were randomly selected for either chow or HFD feeding.

2.2. Real-time PCR

Hypothalamic total RNA was extracted using TRIzol reagent (Invitrogen), according to the instructions provided by the manufacturer. cDNA synthesis was performed using 3.0 μg of total RNA according to the manufacturer's instructions (High-Capacity cDNA Reverse Transcription Kit, Life Technologies). Normalization was obtained by determining the expression of glyceraldehyde-3-phosphate dehydrogenase in all samples. Each PCR contained 25–40 ng of reverse-transcribed RNA, 2.5 μl of each specific primer, Taqman Universal master mix (4369016, Life Technologies) and RNAse-free water at a final volume of 10 μl. Real-time PCR analysis of gene expression was carried out using an ABI Prism 7500 sequence detection system (Applied Biosystems). The following primers were purchased from Applied Biosystems: TNF-α, Mm 99999068_m1; IL-6, Mm99999064_m1; IL-1β, Mm00434228_m1; CX3CL1, Mm00436454_m1; IL-10, Mm01288386_m1; Mfn2, Mm PT 586479342– FAM and GAPDH, 4351309.

2.3. Western blots

Hypothalamus specimens were homogenized in approximately 10 vol of solubilization buffer containing 1% Triton X-100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg/mL aprotinin at 4 °C in a "Polytron PTA 20S Generator" (Brinkmann Instruments mode EN 10/35). Gel loading samples contained 50–125 μg total protein depending on the target protein. Loading was always evaluated by Ponceau staining and reblotting of the membranes with antibodies against housekeeping proteins, α-tubulin or β-actin. The Ponceau stainings of all membranes used to prepare the figures presented in this work are shown in Supplementary Fig. 1. Antibodies employed for blotting were: anti-TNF-α (rabbit polyclonal, SC -8301), anti-IL-1β (goat polyclonal, SC-8481), anti-IL-6 (rabbit polyclonal, SC-7920), IL-10 (goat polyclonal, SC-1783), anti-spliced X-box binding protein 1 (XBP-1, rabbit polyclonal, SC-61950), anti-inositol requiring enzyme 1 alpha (IRE-1α, rabbit polyclonal, SC-20790), anti-pPERK (rabbit polyclonal, SC-32577R), anti-CX3CL1 (fractalkine, rabbit polyclonal, ab-25088), anti-activating transcription factor 6 (ATF6, mouse monoclonal, img-273), anti-G-coupled receptor 78 (GRP78, rabbit monoclonal, ab21685), anti-CCAAT-enhancer-binding protein homologous protein (CHOP, ab11419 monoclonal rabbit), anti-mitofusin-2 (MFN2, rabbit monoclonal, ab-50843), anti-α-tubulin (mouse monoclonal, t-5168) and anti-β-actin (rabbit polyclonal, ab8227). Antibodies were purchased from Santa Cruz Biotechnology, Abcam, Imgenex and Sigma-Aldrich.

2.4. Treatment with 4-phenylbutyrate (PBA) and infliximab

In some experiments, mice were treated with intraperitoenal (ip) injections of either PBA (120 mg/kg) or infliximab (10 mg/kg). The first dose was administered 12 h before the introduction of the HFD, the second dose concomitant with the introduction of the HFD and the third and last dose 12 h later. Experiments were performed 24 h after the introduction of the HFD. PBA was obtained from Sigma-Aldrich and infliximab from Janssen.

2.5. Transmission electronic microscopy (TEM)

For ultrastructural analysis of mitochondria in the arcuate nucleus of the hypothalamus, the animals were subjected to transcardiac perfusion with 0.1 M PBS (20 mL, pH 7.4) and then fixed with 2.5% glutaraldehyde and 1.0% paraformaldehyde in phosphate buffer pH 7.4. The hypothalamus specimens were dissected out and stored overnight in fixative at 4° C. The hypothalamus were then osmicated, dehydrated and embedded in Durcupan ACS (Fluka, Steinheim, Switzerland). Ultrathin cross sections were collected on formvar coated copper grids, contrasted with uranyl acetate and lead citrate, and examined in a Tecnai G2 Spirit Twin (FEI, Hillsboro, OR) transmission electron microscope operated at 80 kV. Mitochondria/endoplasmic reticulum (ER) contacts were blind counted by two independent researchers. Presence of contact was scored positive when ER membrane was clearly touching in tangent with the mitochondria outer membrane.

2.6. Mitochondrial respiration assays

Mice were euthanized and their brains removed rapidly and carefully in order to preserve anatomical references. A fragment of about 2.0–3.0 mg was removed from the mediobasal hypothalamus and placed inside the Oroboros chamber. O_2 consumption was monitored using a Clark electrode operating under continuous stirring at 37 °C, coupled to an Oroboros high-resolution respirometry system. The buffer used contained 150 mM KCl, 10 mM Hepes, 2 mM inorganic phosphate, 2 mM MgCl2, 0.5 mM EGTA, 0.1% bovine serum albumin, and a pH of 7.2 was obtained with the addition of KOH. Specimens were left to rest for 10 min inside the equipment and calibration of the system was obtained. This was followed by the addition of 50 μg/mL saponin to permeabilize the cells, 5.0 mM pyruvate and 3.0 mM malate at saturating concentrations to serve as substrate complex I. After 10 min, 5.0 mM ADP was added at a saturating concentration. Six minutes later, 2.0 μg/mL oligomycin was added; finally, 6 min later 1.0 μM rotenone was added (Herbst and Holloway, 2015).

2.7. Statistical analysis

Results are presented as mean ± standard deviation. For statistical analysis, we apply first the Levene test to evaluate homogeneity. For comparison of means between two groups, we employ a Student's *t*-test for independent samples. When appropriate,

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