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Arachidonic acid impairs hypothalamic leptin signaling and hepatic energy homeostasis in mice

Licai Cheng [a], Yinghua Yu [a,b,*], Qingsheng Zhang [a,b], Alexander Szabo [a,c], Hongqin Wang [a], Xu-Feng Huang [a,b,**]

[a] School of Medicine, Illawarra Health and Medical Research Institute, University of Wollongong, NSW 2522, Australia
[b] Schizophrenia Research Institute (SRI), 405 Liverpool St, Sydney, NSW 2010, Australia
[c] ANSTO Life Sciences, Australian Nuclear Science and Technology Organisation (ANSTO), Lucas Heights, NSW 2234, Australia

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ABSTRACT

Epidemiological evidence suggests that the consumption of a diet high in n-6 polyunsaturated fatty acids (PUFA) is associated with the development of leptin resistance and obesity. We aim to examine the central effect of n-6 PUFA, arachidonic acid (ARA) on leptin sensitivity and leptin-regulated hepatic glucose and lipid metabolism. We found that intracerebroventricular injection of ARA (25 nmol/day) for 2.5 days reversed the effect of central leptin on hypothalamic JAK2, pSTAT3, pAkt, and pFOXO1 protein levels, which was concomitant with a pro-inflammatory response in the hypothalamus. ARA also attenuated the effect of central leptin on hepatic glucose and lipid metabolism by reversing the mRNA expression of the genes involved in gluconeogenesis (G6Pase, PEPCK), glucose transportation (GLUT2), lipogenesis (FAS, SCD1), and cholesterol synthesis (HMG-CoA reductase). These results indicate that an increased exposure to central n-6 PUFA induces central cellular leptin resistance with concomitant defective JAK2-STAT3 and PI3K-Akt signaling.

1. Introduction

Evidence suggests that fatty acids act directly on the central nervous system to affect food intake, insulin sensitivity, and leptin sensitivity (Cintra et al., 2012; Munzberg et al., 2004). Recent studies have shown that the ingestion of a fat-rich diet leads to leptin resistance in the hypothalamus (El-Haschimi et al., 2000; Munzberg et al., 2004). High-fat diets rich in n-6 polyunsaturated fatty acids (PUFA) have been shown to increase the risk of leptin resistance, diabetes, and obesity in humans and rodents (Nuernberg et al., 2011; Phillips et al., 2010). High-fat diet (HFD)-induced leptin resistance and obesity may be mediated by defects in leptin in the Janus kinase 2 and signal transducer and activator of transcription (JAK2-STAT3) and the phosphatidylinositol 3-kinase (PI3K-Akt) signaling pathways in the hypothalamus (Ghilardi et al., 1996; Munzberg et al., 2004; Warne et al., 2011). The JAK2-STAT3 pathway has been shown to mediate the effect of eptin on glucose metabolism (Buettner et al., 2006), and the PI3K-Akt pathway is involved in both glucose (Buettner et al., 2008) and lipid metabolism (Morton et al., 2005). Recent studies have provided strong evidence for the contribution of hypothalamic inflammation to HFD-induced leptin resistance and/or type 2 diabetes mellitus (Posey et al., 2009; Zhang et al., 2008).

A number of studies show that leptin also plays a primary role in the regulation of glucose homeostasis (Denroche et al., 2012). For instance, leptin administration in rodents enhances insulin mediated suppression of hepatic glucose production (Burcelin et al., 1999; Rossetti et al., 1997) and suppresses (German et al., 2011) hepatic gluconeogenesis. Moreover, a large amount of evidence supports direct improving function of leptin in peripheral lipid metabolism by regulating lipogenesis, b-oxidation, and cholesterol metabolism (Gallardo et al., 2007; Prieur et al., 2008). However, the effect of n-6 PUFA on glucose and lipid homeostasis via central leptin way is still unclear.

Arachidonic acid (ARA, 20:4 n-6) is one of the most abundant long-chain PUFA in the brain. It is a key component of cell membranes and serves as a precursor to some eicosanoids (prostaglandins, thromboxanes, leukotrienes) which mediate inflammatory responses (Zhou and Nilsson, 2001). Interestingly, an excess consumption of n-6 PUFA (abundant in the western diet) can increase inflammation and contribute to the pathology of major chronic diseases, including metabolic syndrome, type 2 diabetes mellitus, and obesity (Decsi et al., 1996; Garaulet et al., 2001). Increased ARA content in adipose tissue is also associated with an increased risk of metabolic syndrome and obesity (Garaulet et al., 2001). Finally, the inhibitory role of ARA has been

* Corresponding authors. Illawarra Health and Medical Research Institute, School of Medicine, University of Wollongong, Northfields Avenue, NSW 2522, Australia. Tel.: +61 02 4221 4300; fax: +61 02 4221 8130.
(Y. Yu).
** Corresponding author. Illawarra Health and Medical Research Institute, School of Medicine, University of Wollongong, Northfields Avenue, NSW 2522, Australia. Tel.: +61 02 4221 4300; fax: +61 02 4221 8130.
E-mail address: xhuang@uow.edu.au (X.-F. Huang).

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suggested in both basal and insulin-stimulated leptin expression and production (Nuernberg et al., 2011).

The aim of the present study was to determine the effect of the direct central infusion of ARA on hypothalamic inflammation, leptin sensitivity, leptin signaling, and hepatic energy homeostasis in mice. In addition, to identify possible molecular mechanisms in which ARA is involved, the hypothalamic expression of tyrosine hydroxylase (TH, a sympathetic activity marker) was assessed.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (10 weeks old, body weight: 22.74 ± 3.22 g) were obtained from the Animal Resource Centre (Perth, WA, Australia) and housed in environmentally controlled conditions (temperature 22 °C, 12 hour light/dark cycle). Mice were maintained on a normal lab chow diet (LC; Vella Stock feeds, Doonside, NSW, Australia) throughout the study. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, NSW, Australia, and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

2.2. Experiment protocols

The mice were randomized into four groups: vehicle + saline, vehicle + leptin, ARA + saline, ARA + leptin (n = 12/group). After 1 week of acclimatization, mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An intracerebroventricular (icv) cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) as described in our previous study (Wu et al., 2014). The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track in the brain sections of each animal (Supplementary Fig. S1). A total of 3 times of ARA icv injections (for 2.5 days each time) were conducted in this study.

2.3. Central leptin sensitivity test

Five days after the cannula implantation, a central leptin sensitivity test was performed as described previously (Yu et al., 2013). The mice received icv injections of ARA (25 pmol twice daily for 2.5 days, 5 injections in total) or vehicle (Abizaid and Horvath, 2008). At the end of day 2 of the test, the mice fasted overnight. One hour after the last ARA/vehicle injection, each group of mice will be treated with an icv injection of either leptin (0.50 μg/mice) or saline. Food intake and body weight were measured 24 hours after the icv leptin/saline injection. ARA (A9673, Sigma-Aldrich, Australia) was dissolved in 96% ethanol, dried using nitrogen gas, and then dissolved in 40% hydroxypropyl-b-cyclodextrin (HPB) (H107, Sigma-Aldrich) and stored at –20 °C.

2.4. Intraperitoneal glucose tolerance test (GTT)

After a 3 day interval, icv injections were repeated (details as per "Central leptin sensitivity test"). The glucose tolerance test was performed 30 minutes after the leptin/saline injection. Blood glucose was measured at 0, 30, 60, and 120 min after the glucose administration (0.5 g/kg glucose, intraperitoneal) using a glucometer (Alameda, CA).

2.5. Tissue collection

After another 3 day interval, the icv injections were repeated (details as per "Central leptin sensitivity test"). Thirty minutes after the leptin/saline injection, the mice were sacrificed by CO_2 asphyxiation. The brain and liver were immediately collected, snap frozen in liquid nitrogen, and stored at –80 °C for further processing and analysis. In a cryostat at a temperature of –18 °C, 500 μm frozen brain sections were cut from Bregma –0.58 mm to –2.72 mm according to a standard mouse brain atlas (Song et al., 2008). The mediobasal hypothalamus (MBH) and paraventricular nuclei (PVN) were dissected from frozen coronal sections using a Stoelting Brain Punch (#57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) based on previously described coordinates (Yu et al., 2013).

2.6. Western blot analysis

Western blotting was performed on protein extracts from frozen tissue as described in our previous study (Morton et al., 2005). The expression of specific proteins was determined using the following antibodies: TNF-α (sc-8301), IL-1β (sc-7884), IL-6 (sc-7920), pIκBα (sc-8404), and pJAK2 (sc-21870) (Santa Cruz Biotechnology, California), and pSTAT3 (Tyr705) (#9145), suppressor of cytokine signaling 3 (SOCS3) (#2932), pAkt (#9271), and phosphor-forkhead box protein O1 (pFOXO1) (#9461) (Cell Signaling Technology Beverly, MA, USA). Bands corresponding to the proteins of interest were scanned and band density was analyzed using the Quantity One automatic imaging analysis system (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were normalized to β-actin, based on our previous studies (du Bois et al., 2012). Due to the small amount of tissue in the MBH and PVN of the hypothalamus, we used a previously-described modified multi-strip Western blot, which allows the detection of multiple proteins with a smaller sample size than in a standard Western blot (Yu et al., 2013).

2.7. RNA isolation and RT-PCR

Total RNA from the liver was extracted using the Aurum total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA, USA) and reverse-transcribed to first-strand complementary DNA using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed in a 20 μl final reaction volume using a SYBR green I master on a Lightcycler 480 Real-time PCR System (F. Hoffmann-La Roche Ltd, Switzerland). Amplification was carried out at 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. This was repeated for a total of 45 cycles. The mRNA expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as the internal control. Expression levels for each gene were calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where ΔΔCt = ΔCt sample – ΔCt reference) as described previously (Livak and Schmittgen, 2001). The primers used are listed in Supplementary Table S1.

2.8. Statistics

Data were analyzed using the statistical package SPSS 19.0 (SPSS, Chicago, IL, USA). The two-tailed Student's t-test was used to compare hypothalamic cytokine expression between the ARA and vehicle groups. One-way ANOVA and two-way ANOVA with post hoc Tukey–Kramer honestly significant difference (HSD) test were used to analyze hypothalamic leptin signaling molecules, central leptin sensitivity, and the mRNA expression of genes regulating hepatic glucose and lipid metabolism. $p < 0.05$ was regarded as statistically significant. Values are expressed as mean ± SEM.

3. Results

3.1. Central arachidonic acid administration reduces central leptin sensitivity

To investigate the effect of ARA on central leptin action, we examined food intake and body weight change in response to the

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