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Linoleic acid causes greater weight gain than saturated fat without hypothalamic inflammation in the male mouse

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Abstract

A significant change in the Western diet, concurrent with the obesity epidemic, was a substitution of saturated fatty acids with polyunsaturated, specifically linoleic acid (LA). Despite increasing investigation on type as well as amount of fat, it is unclear which fatty acids are most obesogenic. The objective of this study was to determine the obesogenic potency of LA *vs.* saturated fatty acids and the involvement of hypothalamic inflammation. Forty-eight mice were divided into four groups: low-fat or three high-fat diets (HFDs, 45% kcals from fat) with LA comprising 1%, 15% and 22.5% of kilocalories, the balance being saturated fatty acids. Over 12 weeks, bodyweight, body composition, food intake, calorimetry, and glycemia assays were performed. Arcuate nucleus and blood were collected for mRNA and protein analysis. All HFD-fed mice were heavier and less glucose tolerant than control. The diet with 22.5% LA caused greater bodyweight gain, decreased activity, and insulin resistance compared to control and 1% LA. All HFDs elevated leptin and decreased ghrelin in plasma. Neuropeptides gene expression was higher in 22.5% HFD. The inflammatory gene *Ikk* was suppressed in 1% and 22.5% LA. No consistent pattern of inflammatory gene expression was observed, with suppression and augmentation of genes by one or all of the HFDs relative to control. These data indicate that, in male mice, LA induces obesity and insulin resistance and reduces activity more than saturated fat, supporting the hypothesis that increased LA intake may be a contributor to the obesity epidemic.
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1. Introduction

Obesity in the United States is increasing in prevalence and shows association with many serious, noncommunicable diseases [1]. This has motivated an extensive effort to discover causes of obesity and develop methods for prevention and treatment. The high-fat diet (HFD)-fed mouse is a widely used model for diet-induced obesity (DIO) [2]. Compared to the "cafeteria diet," it provides nutrient consistency, and high-sucrose diets, unless also high in fat [3,4], produce a lean mouse [5,6]. Although recognized as the best DIO model, a better understanding of the influence of fatty acid (FA) profile would aid in discovery of mechanisms and treatments.

Both saturated [7,8] and n-6 polyunsaturated [9,10] fatty acids (SFA and PUFA) have been identified as obesogenic compared to other FAs. Incorrect FA profile reporting may be causing these divergent results. The FA profile of experimental diets is rarely confirmed with gas chromatography but instead reported from a nutrient database. Recent testing by Research Diets (Research Diets, Inc., New Brunswick, NJ, USA) showed that lard, commonly used in HFDs, contains twice the amount of PUFA, mostly as linoleic acid (LA), reported on the USDA National Nutrient Database for Standard Reference Release [11]. Similarly, the FAs consumed by the relevant human population should factor into DIO research. Total fat intake was not greatly changed during the 20th century, and any change in total fat was dwarfed by the increase in PUFA (primarily LA) at the expense of SFA and oleic acid [12]. Accurate reporting of FA profile and integrating epidemiological data are necessary prerequisites to theorizing on mechanisms of obesity.

One candidate mechanism for DIO is hypothalamic inflammation (HI), in which fatty acids play a large part [13–17]. In short, chronic low-grade inflammation impairs neuronal sensing of energy status, resulting in a melanocortin system that acts on downstream neuronal circuits that control feeding and energy expenditure as if adipose storage was insufficient. One proposed HI pathway is activation of Toll-like receptor 4 (TLR4) by SFA [7,18]. Indeed, knockout of TLR4 abrogated DIO from an SFA-rich HFD but, interestingly, not from an LA-rich HFD, which produced greater obesity regardless of TLR4 status [19]. Although important to innate immunity through the recognition of lipopolysaccharide (LPS), TLR4 is not the entirety of the inflammatory system. Central inflammation [20] and metabolic derangement due to HI [21] can be a response to reactive oxidative species. PUFAs are the only fatty acids prone to nonenzymatic oxidation at mammalian body temperatures. Acyl chain carbon–hydrogen bonds

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surrounded by carbon–carbon double bonds are susceptible to nucleophilic attack [22]. Thus, inflammatory mechanisms exist for SFA and PUFA.

Due to the contradictory data on FAs in rodent DIO research, the Western diet being modeled and the involvement of FA in HI, we hypothesized that LA will cause more weight gain and metabolic derangements and greater expression of HI markers than SFA. We fed male WT C57BL6/J mice one of three HFDs with equal fat content but different FA profiles and measured weight, food intake, glucose metabolism, indirect calorimetry and activity. Upon sacrifice, we measured plasma metabolic peptides and biomarker mRNA of HI and metabolism in the arcuate nucleus of the hypothalamus.

2. Material and methods

2.1. Animal care

All animal treatments were in accordance with institutional guidelines based on National Institutes of Health standards and performed with Institutional Animal Care and Use Committee approval at Rutgers University. Male WT C57BL6/J mice were selectively bred in-house, maintained under controlled temperature (23°C) and photoperiod conditions (12-h/12-h light/dark cycle), and given access to food and water *ad libitum*. Mice were weaned and ear-tagged at postnatal day 21 and housed in groups until start of experiment.

2.2. Experimental diets

All experimental diets were prepared as pellets by Research Diets (New Brunswick, NJ, USA). FA profile was assured through in-house gas chromatography/mass spectroscopy. Our control diet (CON) was Research Diets D12450B (10% kcal from fat). There is no logical control diet for FA profile since fat content, unlike a drug, cannot be excluded without the exclusion becoming an experimental variable itself. Therefore, we chose a common control diet as an environmental control and to compare our results to other studies. Our three HFDs were isocaloric and isolipidic to Research Diets D12451 (45% kcal from fat) and named for the amount of calories derived from LA: 1%=mostly coconut oil with some seed oils, 15%= an even mixture of coconut oil and seed oils, and 22.5%=mostly safflower and sunflower seed oils (see Table 1 for FA profile). We chose to maintain a constant n-3 content rather than a constant n-6/n-3 ratio, as this is more similar to the Western dietary change we are modeling. Coconut oil was used for SFA because all other food sources, such as lard, butter or tallow, have significant amounts of n-3 and n-6 PUFA, and comparing synthetic- with food-derived FAs would also confound the interpretation. Although the chain length is probably a factor in obesity, palmitate from tallow has been shown to be less obesogenic than safflower oil [23]. All diets had identical protein, fiber, and micronutrient contents.

2.3. Experimental design

Experimental feeding began at 12 weeks of age. Mice were housed three per cage and given *ad libitum* access to food and water. Body weight and food intake (per cage food intake) were recorded weekly for 12 weeks followed by body composition measurements using an EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) and calorimetric and activity measurements (48-h run) *via* Columbus Instruments' Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Inc., Columbus, OH, USA). A glucose tolerance test (GTT), following an overnight fast, was administered *via* intraperitoneal (IP) injection of 2 g/kg glucose in 0.9% saline solution. Blood glucose (BG) from tail blood was measured with an AlphaTrak 2 Blood Glucose Monitoring System (Abbott Laboratories, Abbott Park, IL, USA) preinjection and 15, 30, 60, 90, 120 and 180 min postinjection. An insulin tolerance test (ITT) following a 5-h fast involved an injection of 0.5 U/kg insulin (Humulin R; Lilly, Indianapolis, IN, USA) in 0.9% saline solution and followed the same BG measurement scheme as GTT. Mice were given 4 days of rest each between CLAMS, GTT and ITT.

2.4. Tissue collection

At completion of physiological assays, mice were given another 4 days of rest while remaining on the same diet and then killed by decapitation after ketamine sedation (100 μl of 100 mg/ml, IP). Trunk blood was collected and prepared for plasma analysis of peptide hormones and cytokines by Luminex Magpix multiplex (EMD Millipore, Billerica, MA, USA). Plasma was prepared by adding a protease inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mg/ml, Sigma-Aldrich), to each K^+ EDTA collection tube. Samples were maintained on ice until centrifugation at 3000 rpm for 10 min at 4°C. Supernatant was then collected and stored at −80°C until analysis. In preparation for RNA extraction and measurement, the basal hypothalamus was cut using a brain slicer matrix (Ted Pella, Inc., Redding, CA, USA) into 1-mm-thick coronal rostral and caudal slices corresponding to plates 42 to 47 and plates 48 to 53, respectively, from The Mouse Brain in Stereotaxic Coordinates [24]. The slices were transferred to RNAlater (Life Technologies, Inc., Grand Island, NE, USA) and stored overnight at 4°C. The rostral and caudal parts of the arcuate nucleus were dissected using a dissecting microscope. The combined arcuate tissue was stored at −80°C. Total RNA was extracted from combined nuclei (rostral and caudal arcuate) using Ambion RNAqueous-Micro Kits (Life Technologies, Inc.) according to the manufacturer's protocol. Using the extraction kits, total RNA was DNase-I-treated, at 37°C for 30 min to minimize genomic DNA contamination. RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA) and an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples with an RNA Integrity Number below 6 were not used.

Table 1
Diet fat compositions

Diets	CON	1%	15%	22.5%
kcal/g	3.85	4.7	4.7	4.7
Oils (g)	45	202.5	202.5	202.5
Coconut oil	0	133	64.5	21.5
Flaxseed oil	0	10	10	10
Lard	20	0	0	0
Safflower oil	0	0	45	45
Soybean oil	25	2	2	2
Sunflower oil	0	57.5	81	124
Carbohydrate (g)	700	255.6	255.6	255.6
Protein (g)	203	203	203	203
% energy from carbohydrate	70	31	31	31
% energy from protein	20	24	24	24
% energy from fat	10	45	45	45
% from SFA	2.26	31	17	8
% from LA	4.22	1	15	22.5
Fatty acids (g)	43.3	199.8	199.9	199.6
C6, caproic	0.0	0.8	0.4	0.1
C8, caprylic	0.0	10.2	5.0	1.7
C10, capric	0.0	7.8	3.8	1.3
C12, lauric	0.0	63.3	30.7	10.2
C14, myristic	0.2	23.9	11.6	3.9
C16, palmitic	6.5	14.0	12.9	11.9
C16:1, palmitoleic	0.3	0.0	0.0	0.0
C18, stearic	3.1	16.9	11.0	7.4
C18:1, oleic	12.6	52.4	51.0	55.8
C18:2, linoleic	18.3	4.7	67.7	101.4
C18:3, linolenic	2.2	5.8	5.8	5.9
C20:4, arachidonic	0.1	0.0	0.0	0.0
C20:5, eicosapentaenoic	0.0	0.0	0.0	0.0
C22:6, docosahexaenoic	0.0	0.0	0.0	0.0
SFA (%)	22.7	69	37.7	18.3
MUFA (%)	29.9	26	25.5	28.0
PUFA (%)	47.4	5	36.8	53.7

2.5. Quantitative real-time polymerase chain reaction (PCR)

cDNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 μl 5× buffer, 25 mM $MgCl_2$, 10 mM dNTP (Clontech Laboratories, Inc., Mountain View, CA, USA), 100 ng random hexamer primers (Promega Corporation, Madison, WI, USA), 40 U/μl Rnasin (Promega) and 100 mM DTT in DEPC-treated water (GeneMate, Bioexpress, Inc., Kaysville, UT, USA) to a total volume of 20 μl. Reverse transcription was conducted using the following protocol: 5 min at 25°C, 60 min at 50°C and 15 min at 70°C. The cDNA was diluted 1:20 with nuclease-free water (GeneMate, Bioexpress) for a final cDNA concentration of 0.5 ng/μl and stored at −20°C. All primers were designed to span exon–exon junctions and synthesized by Life Technologies, Inc., using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 2 for a list of all primer sets used for quantitative real-time PCR (qPCR). For qPCR, 4 μl of cDNA template (an equivalent of 2 ng total RNA) was amplified using either PowerSYBR Green master mix (Life Technologies) or Sso Advanced SYBR Green (BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of BH cDNA in triplicate to determine the efficiency [$E=10^{(-1/m)}-1$, m=slope] of each primer pair. All efficiencies expressed as percent efficiency were approximately equal (one doubling per cycle, 90%–110%). The relative mRNA expression data were analyzed using the $\Delta\Delta C_T$ method [25,26]. Amplification protocol for all the genes was as follows: initial denaturing at 95°C for 10 min (PowerSYBR) or 3 min (Sso Advanced) followed by 40 cycles of amplification at 94°C for 10 s (denaturing) and 60°C for 45 s (annealing) and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10 s, 65°C to 95°C (in increments of 0.5°C) for 5 s and 95°C for 5 s. The Cq geomean of reference genes, *ActB* and *Hprt*, was used to calculate fold change. Quantification values were generated only from samples showing a single product at the expected melting point.

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