Nutrition 31 (2015) 498–507

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Sex-related effects of nutritional supplementation of *Escherichia coli*: Relevance to eating disorders

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ARTICLE INFO

Article history:
Received 21 October 2014
Accepted 26 November 2014

Keywords:
Animal models
Feeding
Body weight
Anxiety
Eating disorders
Anorexia nervosa
Bulimia
Neuropeptides
Microbiota

ABSTRACT

Objectives: The biological background of sex-related differences in the development of eating disorders (EDs) is unknown. Recent data showed that gut bacteria *Escherichia coli* induce autoantibodies against anorexigenic α-melanocyte-stimulating hormone (α-MSH) associated with psychopathology in ED. The aim of this study was to compare the effects of *E. coli* on feeding and autoantibodies against α-MSH and adrenocorticotropic hormone (ACTH), between female and male rats.

Methods: Commensal *E. coli* K12 were given in a culture medium daily to adult Wistar rats by intragastric gavage over a 3-wk period; control rats received culture medium only.

Results: Before gavage, *E. coli* K12 DNA was detected in feces of female but not male rats. *E. coli* provision was accompanied by an increase in body weight gain in females, but a decrease in body weight gain and food intake in males. Independent of *E. coli* treatment, plasma levels of anti-α-MSH and ACTH immunoglobulin (Ig)G were higher in female than male rats. Females responded to *E. coli* by increasing α-MSH IgG levels and affinity, but males by increasing α-MSH IgM levels. Affinity of IgG for ACTH was increased in both *E. coli*-treated females and males, although with different kinetics. IgG from females stimulated more efficiently α-MSH-induced cyclic adenosine monophosphate production by melanocortin 4 receptor-expressing cells compared with IgG from males.

Discussion: Sex-related response to how *E. coli* affects feeding and anti-melanocortin hormone antibody production may depend on the presence of these bacteria in the gut before *E. coli* supplementation. These data suggest that sex-related presence of certain gut bacteria may represent a risk factor for ED development.

Introduction

The main types of eating disorders (EDs), anorexia nervosa (AN) and bulimia, occur about 10 times more frequently in females than in males [1]. Although the eating behavior and metabolism are physiologically influenced by sex hormones [2,3], their role in the sex-related prevalence of EDs is unknown. The present study aimed to clarify the biological background of a sex-dependent risk factor for ED by exploring sex-related effects of gut bacteria on feeding and production of autoantibodies (autoAbs)-reactive with the host peptide hormones controlling appetite.

Melanocortin peptides, α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) are products of the same pro-opiomelanocortin (POMC) precursor and are critically involved in the regulation of energy balance via binding

This study received support from the EU INTERREG IVA 2 Seas Program (7-003-FR_TC2 N) and the Region of Haute Normandie, France. The authors have no conflicts of interest to declare.

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http://dx.doi.org/10.1016/j.nut.2014.11.003
0899-9007/

to melanocortin receptor type 4 (MC4 R) resulting in increased satiation [4] and thermogenesis [5]. Indeed, in both humans and mice, gene mutations of POMC [6,7] and MC4 R [8,9] lead to overeating and obesity. Furthermore, MC4 R is involved in signaling mood and emotion [10,11]. ACTH is also a major mediator of the hypothalamic–pituitary–adrenal (HPA) axis, acting via MC2 R in the adrenal cortex to increase cortisol secretion in response to stress.

Anorexia nervosa and bulimia are characterized by altered feeding behavior, anxiety, depression, and altered stress response [12], indicating that the melanocortin system may be potentially involved in the ED pathophysiology. However, no significant associations have been found between genetic variants of MC4 R and AN [13], bulimia [14] or binge-eating [15], suggesting alternative mechanisms of altered melanocortin signaling in ED. One such possibility may involve immunoglobulins (Ig), that is, autoAbs that can modulate biological effects of peptide hormones (e.g., by increasing their stability) [16].

Indeed, it was previously shown that in both humans and rats, circulating IgG may bind to α-MSH and ACTH [17,18] and that plasma levels of these autoAbs correlate with some typical behavioral traits in female patients with EDs [19]. Furthermore, α-MSH autoAbs were associated with anxiety and depression in healthy individuals [20] and ACTH autoAbs with antisocial behavior in adolescents [21], suggesting that autoAbs reactive with the melanocortin hormones may physiologically regulate behavioral traits in humans. It was also shown that the repeated mild stress in rats changed binding properties of α-MSH autoAbs, linking them to the control of food intake and anxiety [22].

Regarding the mechanisms underlying the origin of autoAbs against melanocortin hormones, our recent work showed that α-MSH-reactive autoAbs can be stimulated by homologous to α-MSH antigenic proteins produced by commensal gut bacteria. In fact, we identified that the heat-shock ClpB protein of *Escherichia coli* is a conformational mimetic of α-MSH and that providing *E. coli* to male mice increased plasma levels of anti-ClpB IgG cross-reactive α-MSH [23]. Furthermore, correlations between ClpB IgG levels and psychopathologic traits found in ED further supported that ClpB-containing bacteria may be involved in the etiology of ED [23]. It is, hence, possible that there might exist sex-related differences in response to *E. coli* to produce autoAbs against α-MSH and ACTH.

Thus, in the present work, we tested our hypothesis that *E. coli* nutritional supplementation may influence production of α-MSH and ACTH autoAbs differently in male and female rats associated with changes in feeding and anxiety.

Materials and methods

Animals

Animal care and experimentation were in accordance with guidelines established by the U.S. National Institutes of Health, and complied with both French and European community regulations (*Official Journal of the European Community* L 358, 18/12/1986). Male and female Wistar rats (N = 48), body weight 220 to 250 g, were purchased from Janvier Labs (Le Genest-St-Isle, France) and were maintained at 24°C with a 12-h light–dark cycle in a specialized animal facility. The rats were fed with standard pelleted rodent chow (RM1 diet, SDS, UK) when kept in standard holding cages for 1 wk. After acclimation, rats were transferred to individual metabolism cages (Tecniplast, Lyon, France) where they were fed with the same RM1 but grounded chow (SDS). Drinking water was always available. Body weight and intake of food and water were measured daily. Male and female rats were studied in two separate but identical experiments. Although the estrous cycle is known to influence meal pattern in female rats [24], in the present study it was not taken into consideration because total food intake was measured and the study duration included more than four to five cycles. The day before starting the gavage, blood samples (200 μL) were taken by the retro-orbital puncture in rats anesthetized by an intraperitoneal (IP) injection (25 mg/kg body weight) of Brietal (Lilly, Nieuwegein, Netherlands). Plasma was separated by centrifugation at 3500*g* for 10 min at 4°C and stored at −80°C before assay.

E. coli *K12 culture and gavage protocols*

The bacterial strain used in this study was *E. coli* K12. This strain was available from the UMR 6270 CNRS Laboratory in Rouen University, France. From the frozen stocks (−80°C), *E. coli* K12 were suspended in 250 mL of Luria broth (LB) liquid medium (MP, Illkrich, France) and plated in LB agar to detect the contaminations. The medium containing bacteria were cultured anaerobically at 37°C for 24 h in 50-mL tubes. To estimate the number of *E. coli* bacteria, optical density (OD) of bacterial culture was measured by a spectrophotometer at λ = 600 nm with OD 0.1 corresponding to about 10^8 cells/mL. Rats were intragastrically gavaged with 4 mL of LB medium containing 10^8 *E. coli* K12 (n = 12) or with 4 mL of LB medium without bacteria (controls, n = 12). The gavage procedure was carried out daily between 0900 and 1000 for 21 d.

E. coli *K12 bacterial DNA assay*

The DNA was purified from feces of males and females rats with the QIAamp^R DNA Stool Mini Kit (QIAGEN, France), DNA of *E. coli* K12 was also extracted from the cultures of the strain; bacteria were dissolved in water and warmed at 100°C for 5 min, after 1 min of centrifugation at 11000*g*, the supernatant containing the DNA was stored at −20°C.

Based on the published study [25], we used the following nucleotide primers, K12-R: 5′-ATCCTGCGCACCAATCAACAA-3′ and K12-F: 5′-CGCGATGGAA GATGCTCTGTA-3' (Invitrogen Custom Primers, Cergy Pontoise, France) to amplify a region of the orf264 as a marker of *E. coli* K12. Polymerase chain reaction was performed in a thermocycler with MicroAmp tubes (Eppendorf, Hambourg, Germany). The reaction was carried out in a 50-μL volume containing 25 μL of Go Taq ^{R} Green Master Mix 2 X (Promega, Madison, WI), 1 μL (20 pmol) of each primer, 21 μL of bi-distilled water and 1 μL of bacterial DNA. PCR conditions were as follows: 3 min at 94°C followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1.5 min. PCR products were analyzed on a 1% agarose gel (Sigma, St Louis, MO, USA), the size of the amplification product corresponding to 0.97-kb.

Plasma autoantibody assay

Plasma levels of IgG or IgM autoAbs reacting with α-MSH, ACTH (IgG only) and ClpB were measured using enzyme-linked immunosorbent assay (ELISA) technique according to a published protocol [26]. Briefly, α-MSH or rat ACTH peptides (Bachem AG, Bubendorf, Switzerland) or *E. coli* ClpB (Delphi Genetics SA, Gosselies, Belgium) were coated on Maxisorp plates (Nunc, Rochester, NY, USA) using 100 μL and a concentration of 2 μg/mL in 100 mM $NaHCO_3$ buffer, pH 9.6 for 72 h at 4°C. Plates were washed (5 min × 3) in phosphate-buffered saline (PBS) with 0.05% Tween 200, pH 7.4, and then incubated overnight at 4°C with 100 μL of rat plasma diluted 1:200 in PBS to determine free autoAbs levels or diluted 1:200 in dissociative 3 M NaCl, 1.5 M glycine buffer, pH 8.9 to determine total autoAbs levels. The optimal dilutions of plasma were determined as 1:200. The plates were washed (3 ×) and incubated with 100 μL of alkaline phosphatase-conjugated goat anti-rat IgG (1:2000) or antirat IgM (1:1000) both from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). Following washing (3 ×), 100 μL of *p*-nitrophenyl phosphate solution (Sigma) was added as alkaline phosphatase substrate. After 40 min of incubation at room temperature, the reaction was stopped by adding 3 N NaOH. The OD was determined at 405 nm using a microplate reader Metertech 960 (Metertech Inc., Taipei, Taiwan). Blank OD values resulting from the reading of plates without addition of rat plasma were subtracted from the sample OD values. Each determination was done in duplicate. The variation between duplicate values was <5%. Plasma concentrations of total IgG and IgM were measured using Rat IgG and IgM ELISA Quantitation Sets (Bethyl Laboratories Inc., Montgomery, TX, USA).

IgG purification from plasma

Purification of plasma IgG for the analysis of their affinity was done according to a published protocol [27]. Plasma globulins were separated on C-18 SEP columns (Phoenix Pharmaceuticals, Burlingame, CA, USA) after plasma acidification by mixing 500 μL of rat plasma was 500 μL of buffer A (1% trifluoroacetic acid [TFA] in water). The column was activated in 1 mL of buffer B (60% acetonitrile in 1% TFA) by 3 min centrifugation with 700*g* and rinsed 3 times with 3 mL of buffer A. Diluted plasma (1:1 in buffer A) was added to the column and the effluent (1 mL) was saved and frozen at −20°C. Total IgG were purified from the effluents of rat plasma samples using the Melon Gel Kit (Thermo Fisher Scientific, Rockford, IL, USA). In brief, plasma effluents diluted 1:4 in the kit's purification buffer was added on washed melon gel deposited in a column. Column was spinned