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Protein malnutrition after weaning disrupts peripheral clock and daily insulin secretion in mice☆

Patricia Cristine Borck[a], Thiago Martins Batista[a], Jean Franciesco Vettorazzi[a], Rafael Ludemann Camargo[a], Antonio Carlos Boschero[a], Elaine Vieira[b,*,1], Everardo Magalhães Carneiro[a,1]

[a]Department of Structural and Functional Biology, Institute of Biology, University of Campinas/UNICAMP, Campinas, SP, Brazil
[b]Postgraduate Program on Physical Education, Universidade Católica de Brasília-UCB, Brasília, DF, Brazil

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Abstract

Changes in nutritional state may alter circadian rhythms through alterations in expression of clock genes. Protein deficiency has a profound effect on body metabolism, but the effect of this nutrient restriction after weaning on biological clock has not been explored. Thus, this study aims to investigate whether the protein restriction affects the daily oscillation in the behavior and metabolic rhythms, as well as expression of clock genes in peripheral tissues. Male C57BL/6 J mice, after weaning, were fed a normal-protein (NP) diet or a low-protein (LP) diet for 8 weeks. Mice fed an LP diet did not show difference in locomotor activity and energy expenditure, but the food intake was increased, with parallel increased expression of the orexigenic neuropeptide *Npy* and disruption of the anorexigenic *Pomc* oscillatory pattern in the hypothalamus. LP mice showed disruption in the daily rhythmic patterns of plasma glucose, triglycerides and insulin. Also, the rhythmic expression of clock genes in peripheral tissues and pancreatic islets was altered in LP mice. In pancreatic islets, the disruption of clock genes was followed by impairment of daily glucose-stimulated insulin secretion and the expression of genes involved in exocytosis. Pharmacological activation of REV-ERBα could not restore the insulin secretion in LP mice. The present study demonstrates that protein restriction, leading to development of malnutrition, alters the peripheral clock and metabolic outputs, suggesting that this nutrient provides important entraining cues to regulate the daily fluctuation of biological clock.

Keywords: Clock genes; Insulin secretion; Locomotor activity; Peripheral tissues; Protein restriction

1. Introduction

Circadian rhythms in behavior and metabolism are regulated by internal biological clock [1,2]. In mammals, they are mainly controlled by the suprachiasmatic nucleus (SCN), which interacts with the peripheral clock, coordinating the cyclic oscillations in hormones and metabolic pathways [1]. Disturbances in the clock network alter glucose and lipid homeostasis, leading to the development of metabolic diseases such as obesity and diabetes [3–6].

At the molecular level, the core circadian clock comprises the transcription factors of circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1), which bind to E-box enhancers in the *Period* (*Per*) and C*ryptochrome* (*Cry*) promoters. The PER and CRY protein complex inhibits its own CLOCK:BMAL1-induced transcription, and the turnover of PER and CRY allows this cycle to continue. Some nuclear receptors, such as the reverse erythroblastosis virus α (REV-ERBα), can negatively regulate *Bmal1*, whereas the retinoic-acid receptor-related orphan receptor α (RORα) positively regulates its expression. Both central and peripheral biological clocks can be reset by feeding/fasting cycles and metabolic signals, resulting in a clear link between nutrient signaling and circadian transcription [7–13].

The nutritional state during the fetal and postnatal periods has a profound impact on health. It has been reported that the protein restriction during the gestational period and at early life leads to alteration in metabolic and hormones responses, resulting in lower body weight, improvement of glucose tolerance and peripheral insulin sensitivity, as well as reduction in the glucose-stimulated insulin secretion (GSIS) [14–18]. The impact of protein restriction on circadian rhythms has been investigated in offspring born from dams fed with low-protein diet. Chronic low-protein diet during pregnancy in rats altered the circadian system by inducing a lack of coupling among oscillators due to the neuronal changes observed in SCN [19]. Furthermore, offspring that underwent a maternal protein

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* Corresponding author at: Postgraduate Program on Physical Education, Universidade Católica de Brasília-UCB, QS 07 Lote 1, Águas Claras, Brasília, DF 71966-700, Brazil. Tel.: +55 61 33569234.
E-mail address: elaine.vieira@ucb.br (E. Vieira).
[1] These authors contributed equally to the present paper.

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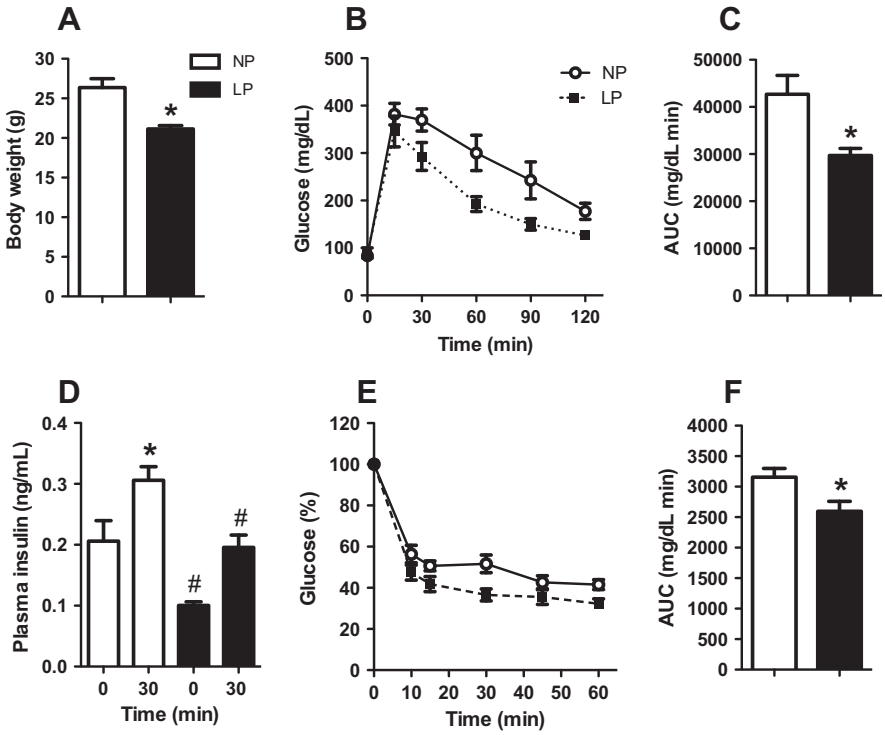

Fig. 1. Low-protein diet leads to lower body weight and improvement of glucose tolerance and insulin sensitivity. (A) Body weight. Changes in blood glucose during IpGTT (B) and IpITT (E) in mice subject to chow diet (NP) and LP diet for 8 weeks. Total plasma glucose concentration during IpGTT (C) and IpITT (F). Plasma insulin levels at 0 min and at 30 min of IpGTT (D). Data are means±S.E.M. (n=7–8). *P<.05 (NP vs. LP AUC, and NP 0 min vs. NP 30 min). #P<.01 (NP 0 min vs. LP 0 min, and NP 30 min vs. LP 30 min). Student's t test.

restriction had alterations in the circadian expression pattern of clock and clock-controlled genes in liver and hypothalamus [20,21]. In addition, previous studies have reported that different dietary protein content, at early and adult life, alters the circadian plasma levels of gastrointestinal peptides, insulin and glucagon, as well as the daily oscillation of hepatic cyclic AMP [22,23]. Although, the relationship between changes in dietary protein content during different life stages and disturbances in the circadian rhythms has been established, the effect of protein restriction on behavioral and metabolic rhythms and clock gene expression is still unknown. Thus, the aim of this study was to determine whether protein restriction after weaning affects energy homeostasis and the daily oscillation in GSIS and expression of clock genes in peripheral tissues.

2. Materials e methods

2.1. Animals and experimental design

All experimental procedures were approved by the Institutional Ethical Committee at UNICAMP (ethical number: 2938-1), and the methods were conducted in accordance with the approved guidelines. After weaning, male C57BL/6 J mice weighing between 13 and 15 g were obtained from breeding colony at UNICAMP (Campinas, Brazil) and divided randomly into two groups according to their isocaloric diets: normal-protein diet (NP; 14% protein) or low-protein diet (LP; 6% protein). The composition of these diets was described in our previous publication [24]. Mice were kept in a 12:12 h light/dark cycle at a temperature 22°C and with free access of food and water. After 8 weeks, the mice were euthanized in a chamber with CO_2 at different times of the day (0800, 1400, 2000 and 0200 h) which correspond to Zeitgeber times (ZTs) 2, 8, 14 and 20, respectively, and blood and tissues samples were collected and snap frozen at −80°C.

2.2. Biochemical analysis

Blood samples from fed mice were used to measure the total plasma protein (LaborClin, Pinhais, Brazil), triglycerides (TGs), cholesterol (Roche, Mannheim, Germany) and plasma albumin (BioClin, Belo Horizonte, Brazil). Plasma insulin was analyzed by radioimmunoassay (RIA) using ^{125}I-labeled human insulin (PerkinElmer, Waltham, MA, USA), rat insulin as standard (Crystal Chem Inc., Downers Grove, IL, USA) and rat insulin antibody. The charcoal-dextran method was performed to separate free insulin from antibody-bound [^{125}I]insulin.

2.3. Metabolic in vivo studies

After an overnight fast (12 h), the glycemia was measured by a glucometer (Accu-Chek Advantage; Roche, Mannheim, Germany). Then, a glucose load of 2 g/kg was administered intraperitoneally, and the glycemia was measured at subsequent intervals of 15, 30, 60 and 120 min. For ipITT, fed mice received 0.75 IU/kg body weight of human recombinant insulin intraperitoneally (Humulin, Indianapolis, IN, USA). Blood samples were collected before and at 5, 10, 15, 30 and 60 min after insulin administration for glucose analysis. In both tests, the area under the curve (AUC) was calculated.

The mice were individually placed in the chambers and allowed to adapt for 24 h. After that, respiratory exchange ratio (RER) and energy expenditure were monitored for 24 h using a calorimeter Oxylet system (Pan Lab/Harvard Instruments, Barcelona, Spain). For food intake, the mice were individually placed in the chamber, and after 24 h adapting, the food consumption was measured at ZTs 2, 8, 14 and 20.

2.4. Circadian locomotor activity

The mice were individually placed in the chambers containing an optical beam sensor system and allowed to adapt for 24 h. After that, the locomotor activity was measured for 48 h using the PheCOM system (Pan Lab/Harvard Instruments).

2.5. Islet isolation and insulin secretion

After 8 weeks of NP or LP diet, at ZTs 2, 8, 14 and 20, the islets were isolated by digestion of the pancreases with collagenase V (0.8 mg/ml). After the digestion, the islets were divided into two groups. For the first one, the islets were collected for gene expression analysis, and for the second group, the islets were used to measure the insulin secretion. To perform the GSIS, groups of four fresh islets were first incubated for 30 min at 37°C in Krebs–Ringer bicarbonate buffer with 5.6 mmol/L glucose and 3 g/L bovine serum albumin, and equilibrated with a mixture of 95% O_2/5% CO_2 to achieve a pH of 7.4. This medium was then replaced with fresh buffer, and the islets were incubated for 1 h in the presence of 2.8, 5.6, 16.7, 22.2, 27.7 and 33.3 mmol/L glucose. Aliquots of the supernatant at the end of the incubation period were kept at −20°C for