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Regulation of corticosterone function during early weaning and effects on gastric cell proliferation

Heloisa Ghizoni M.S. [a], Priscila Moreira Figueiredo M.S. [a], Marie-Pierre Moisan Ph.D. [b,c], Daniela Ogias Ph.D. [a], Luciana Harumi Osaki Ph.D. [a], Patrícia Gama Ph.D. [a,*]

[a] Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil
[b] INRA, Nutrition et neurobiologie intégrée, UMR 1286, Bordeaux, France
[c] Univ Bordeaux, Nutrition et neurobiologie intégrée, UMR 1286, Bordeaux, France

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ABSTRACT

Objectives: The development of the gastrointestinal tract depends on many elements, including glucocorticoids. In the current study, we evaluated the effects of early weaning on corticosterone function and the growth of rat gastric mucosa.
Methods: By using Wistar rats submitted to early weaning at 15 d, we analyzed plasma corticosterone, corticosteroid-binding globulin (CBG), and glucocorticoid receptor (GR) distribution in the gastric epithelium.
Results: With the use of radioimmunoassay, we found that early weaning increased corticosterone concentration at day 16 and 17 in test subjects as compared with controls, whereas it was equivalent between groups at day 18. CBG binding capacity decreased during treatment, and it was significantly lower at day 18. At this age, GR levels and distribution in the gastric mucosa were also reduced as compared with suckling counterparts. To reduce corticosterone activity during early weaning and to explore cell proliferation responses, we administered RU486 to 15-d-old pups. We found that cytoplasmic GR reached a peak after 48 h, whereas nuclear levels remained constant, thereby confirming the inhibition of receptor function. Next, by checking gastric proliferative responses, we observed that RU486 induced higher DNA synthesis and mitotic indices in test subjects as compared with control groups.
Conclusions: We demonstrated that early weaning changed corticosterone activity by increasing hormone levels, reducing CBG binding capacity, and decreasing GR distribution in the gastric epithelium. These modifications seem to be important to the reorganization of gastric growth after the abrupt interruption of suckling.

Introduction

Interactions among dietary conditions, feeding patterns, luminal microbiota, and the synthesis and activity of hormones and growth factors regulate the ontogenesis of the gastrointestinal tract [1–7]. When this mechanism is disturbed, the genetic programming of growth is altered, which induces morphophysiologic changes that modify the function of organs during adult life [8,9]. Among postnatal adversities, early weaning represents the abrupt substitution of milk by solid food intake in combination with restricted maternal contact [1,10,11].

In the rat gastric epithelium, early weaning increases cell proliferation and differentiation [1,4,5,12]. More specifically, the dietary change induces extracellular signal-regulated protein kinases 1 and 2 phosphorylation that affects the cell cycle [13] and that stimulates proliferation [1,5], and it also increases the synthesis of mucin 6, which is an important marker of gastric maturation [4]. The precocious function of brush border enzymes has also been described [10] as being concomitant with the malformation of epithelial barrier [14] and the lower expression

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* Corresponding author: Tel.: +55-11-3091-7303; fax: +55-11-3091-7402.
E-mail address: patgama@usp.br (P. Gama).

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of alkaline phosphatase in the small intestine [15]. Thus, early weaning disrupts important steps of gastrointestinal tract development, which suggests that the natural transition from suckling to weaning can be essential to the establishment of digestive and metabolic functions [1,4,5,10,12,14,15].

Early weaning triggers different neuroendocrine and behavioral responses [8,16], but the changes in feeding patterns are equally important for the control of the hypothalamic–pituitary–adrenal axis [3,12,17]. Corticosterone is the main glucocorticoid hormone in the rat, and it gradually increases between postnatal days 14 and 24 in parallel with the maturation of organs and systems [18,19].

Glucocorticoid (GC) availability and activity are regulated by different factors, such as corticotrophin-releasing hormone, adrenocorticotropic hormone, 11β-hydroxysteroid dehydrogenases (type 1 and type 2), corticosteroid-binding globulin (CBG), and glucocorticoid receptor (GR) [20–23]. CBG is a transcortin glycoprotein produced by the liver that binds with high affinity to plasma GC [24] and reduces its availability to tissues. Thus, CBG creates a protection mechanism against high corticosteroid levels, but it also provides a reservoir of readily available GC [20,25,26]. Free GC triggers effects within cells through intracellular receptors (i.e., GRs) that can act as transcription factors. Stressful stimuli modulate GR expression, distribution, phosphorylation, and function in many organs in parallel with corticosterone and CBG variation [3,23,27].

Previous reports have demonstrated that fasting increases corticosterone and that corticosterone differentially controls the binding of CBG and GR activity in the rat gastric epithelium, which contribute to the regulation of epithelial cell cycle in pups and adults [3,17]. Other studies have shown that the abrupt dietary changes induced by early weaning increase transforming growth factor-α and epidermal growth factor receptor expression in the stomach as well as signaling through the mitogen-activated protein kinase cascade [4], which are involved in the proliferative and maturation stimuli detected at the beginning of treatment [1,4].

Our hypothesis was that early weaning may change corticosterone activity that would locally be part of the complex mechanism that controls gastric growth. Thus, our aim was to evaluate how early weaning influenced corticosterone and whether hormone responses modified cell proliferation in the gastric epithelium of developing rats. We first examined the effects on plasma corticosterone levels, CBG binding capacity, and GR distribution in the gastric mucosa of rat pups. To explore whether the changes would be functionally significant to gastric cell proliferation, we used RU486 to reduce GR translocation into the cell nucleus and studied the effects promoted by such treatment.

Materials and methods

Animals and early weaning

Wistar rats were obtained from the Animal Colony at the Department of Cell and Developmental Biology (ICB USP). Experiments were approved by the Ethical Committee for Animal Use (CEUA protocols 86/2008; 80/2011). Pregnant females were kept in individual cages, and delivery was set as d 0. Litters were culled to 8 to 9 pups per dam around day 3; they were kept at 22°C and with a schedule involving 12 h of light and 12 h of dark (lights went on at 0600).

At 15 d, pups from different litters were divided into two groups: suckling control (S) and early weaning (EW). Control animals were kept with the dam to suckle freely until euthanasia, whereas EW rats were separated, housed in small plastic cages, and fed with hydrated powdered chow and water ad libitum (Nuvilab CR1, Nuvital Nutrients SA, PR, Brazil). Twice a day, water and food were offered to EW pups, which were also stimulated to eliminate urine and feces after abdominal massage. To mimic such handling, control animals were manipulated daily. Body mass was monitored throughout the experimental period.

Plasma collection and total corticosterone assay

From postnatal days 15 to 18, S and EW rats were anesthetized with isoflurane (Cristália, São Paulo, SP, Brazil) for blood collection from the abdominal aorta. This procedure was conducted within 3 min to avoid the increase of corticosterone levels caused by handling [3]. Blood was collected into heparinized tubes, which were centrifuged (12000 rpm for 20 min), and the plasma was frozen (−20°C). To prevent circadian variations, all euthanasia procedures were conducted at 1700. At least 4 pups/group/age were analyzed, and both males and females were used; previous studies did not report sex differences during this week of postnatal development [28,29].

Serum levels of total corticosterone were assessed via radioimmunoassay using a rat ^{125}I corticosterone kit (ICN Biomedicals, Costa Mesa, CA, USA) according to the manufacturer's instructions. The amount of radioactivity was evaluated in a γ-scintillation counter (Wizard Automatic Gamma Counter, PerkinElmer, Waltham, MA, USA). The intra-assay coefficient variation was 6.2%, and the inter-assay coefficient variation was 11.6%.

Corticosteroid-binding globulin binding assay

CBG was measured with the use of a saturation binding assay that was previously described for pups [3]. Plasma samples were diluted 1:50 and incubated with dextran-coated charcoal suspension (Merck, Darmstadt, Germany). The dextran-coated charcoal suspension was then precipitated by centrifugation, and an aliquot of the samples was added into duplicate tubes that contained different concentrations (0.5–16 nM) of corticosterone-[1,2-^{3}H] (specific activity: 79 Ci/mmoL; GE Healthcare, Buckinghamshire, UK) and into one tube containing 32 μM of cold corticosterone for the evaluation of nonspecific binding. Cold dextran-coated charcoal suspension was added to remove unbound steroids. The reaction was terminated by centrifugation (3000 rpm, 15 min, 4°C). Supernatants were transferred into scintillation vials and counted (TriCarb 1600TR Liquid Scintillation Counter, PerkinElmer). Specific binding was determined and converted into nM of serum.

Stomach collection

Rat pups were euthanized on postnatal days 15, 16, 17, and 18 after anesthesia with a 1:1 (v/v) mixture of xylazine (Rompun; Bayer, São Paulo, Brazil) and ketamine chlorhydrates (Ketamina; Agener, São Paulo, Brazil) (0.5 mL/100 g body weight). Their stomachs were immediately excised, opened along the small curvature, and submitted to either mucosal scraping or fixation in 10% formaldehyde.

Immunoblot for glucocorticoid receptor

After scraping the mucosa of the corpus region, tissue was either collected into cold Tris-buffered saline with 10 mM of phenylmethylsulfonyl fluoride (Merck) or frozen in liquid nitrogen for total and cellular extracts, respectively. Protein lysates were homogenized with a radioimmunoprecipitation buffer (150 mM sodium chloride, 1% NP-40, 1% sodium deoxycholate in 50 mM Tris-hydrochloride, pH 7.5) that contained a cocktail of inhibitors for proteases and phosphatases (1 mM phenylmethylsulfonyl fluoride, 0.45 mg/mL benzamidine, 1 mM leupeptin, 1 mM aprotinin and 5 mM sodium orthovanadate; Sigma, St. Louis, MO, USA). Concentration was determined via the Bradford method [30]. Cytoplasmic and nuclear extracts were separated with the use of nuclear protein extraction and bicinchoninic acid kits (Pierce, Rockford, IL, USA) [3]. To control the quality of separation, 5 μL of each fraction were immunoblotted for specific proteins, and samples that were not properly isolated were excluded. All vials were frozen at −80°C.

Thirty micrograms of total protein, 15 μg of cytoplasmic extracts, and 5 μg of nuclear extracts were separated into 12% sodium dodecyl sulphate-polyacrilamide gel electrophoresis. Samples were electroblotted to nitrocellulose membranes (Hybond-ECL, GE Healthcare), which were washed in Tris-buffered saline and blocked with such saline that also contained 0.1% Tween 20 and 5% nonfat dry milk (1 h, room temperature). Membranes were incubated overnight (4°C) with antibodies against GR (1 μg/mL, M20, Santa Cruz Biotechnology, CA, USA), β-actin (0.7 μg/mL, Sigma), α-tubulin (0.8 μg/mL, Santa Cruz Biotechnology) and lamin-B1 (0.7 μg/mL) (ABCAM, Cambridge, MA, USA). Immunoblots were developed with the ECL Kit (GE Healthcare), and signals were registered with x-ray films (MXG-Plus, Kodak, São Paulo, Brazil). Densitometry was performed with Image J software version 1.37 (National Institutes of Health, public domain, USA).

Immunohistochemical localization of glucocorticoid receptor

GR was detected in the mucosa as described previously [3]. Briefly, nonserial 6-μm sections were deparaffinized, rehydrated, and blocked for peroxidase activity and nonspecific binding. After antigen retrieval (10 mM citric acid, pH 6.0 in microwave), sections were incubated with anti-GR (M20, 1 μg/mL, overnight, 4°C,