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Energy-restricted diet during lactation programs ovarian growth factor and gonadotropin receptor expression in rats

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Abstract

The goal of this study was to evaluate whether a maternal energy-restricted diet could program ovarian angiogenesis and growth factor and gonadotropin receptors expression in rats. After parturition, six Wistar rats with 6 pups each were randomly assigned to one of the following groups: control group (C), with free access to a standard laboratory diet containing 23% protein; and energy-restricted group (ER), receiving 60% of the amount consumed by the control group. After weaning, all female pups had free access to the standard laboratory diet until 90 days of age, when they were sacrificed during the proestrus stage. Blood was collected for further evaluation of serum estradiol levels. One ovary was stored at -80ºC for RT-PCR analysis while the other one was paraffin embedded, sectioned at 5-µm thickness and processed by routine histological analysis for evaluation of vessel density. The ER group had a reduction in follicle stimulating hormone (C = 2.36 ± 0.21, ER = 0.97 ± 0.15, $P < 0.001$) and luteinizing hormone (C = 1.81 ± 0.11, ER = 0.74 ± 0.11, $P < 0.0004$) receptor expression. This group also had an increase in gene expression of basic fibroblast growth (C = 0.51 ± 0.07, ER = 0.72 ± 0.06, $P < 0.04$) and vascular endothelial growth factors (C = 0.82 ± 0.03, ER = 1.06 ± 0.08, $P < 0.02$). Estradiol serum concentration, vessel density, kinase-insert domain receptor, Fms-like tyrosine kinase-1 and fibroblast growth factor receptor-1 gene expressions were unchanged. The results indicated that a maternal energy-restricted diet during lactation programs the ovarian response to FSH, LH and angiogenic factors.

Keywords: angiogenesis, follicle stimulating hormone, lactation, luteinizing hormone, malnutrition, ovary, rat.

Introduction

Among the many endothelial regulators, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been characterized as potent promoters of angiogenesis (Redmer and Reynolds, 1996). VEGF120 and VEGF164 are expressed in the mammalian ovary and are associated with follicular angiogenesis during follicular development (Shimizu *et al*., 2003). Basic FGF is important in regulating ovarian functions including steroidogenesis (Vernon and Spicer, 1994) and initialization of follicular development (Nilsson *et al*., 2001). The best-characterized VEGF receptors are VEGFR1 or Flt-1 (Fms-like tyrosine kinase-1) and VEGFR2 or Flk-1 (fetal liver kinase)/kinase-insert domain receptor (KDR; Ferrara and Davis-Smyth, 1997). The FGF receptor, FGFR1, is highly expressed in small microvessels at all stages of follicular development, and in large microvessels, especially in the late luteal stage of follicular development (Reynolds and Redmer, 1998)

Early food restriction can change the original programming of organs, especially those in developmental phases, which can result in long-term changes in metabolism (Lucas, 1994). Recently, it has been shown by our group that a maternal protein-energy restricted diet during lactation programs the reproductive system of the offspring by altering folliculogenesis, the expression of leptin and its isoform receptors (Cavalcante *et al*., 2009), gonadotropin receptor expression (FSHR - follicle stimulating hormone receptor; LHR - luteinizing hormone receptor), aromatase enzyme expression (Faria *et al*., 2010) and VEGF and FGF receptor expression (Ferreira *et al*., 2010). Similarly, only an energy restricted diet during lactation affected folliculogenesis and the expression of leptin and its isoform receptors (Cavalcante *et al*., 2009).

The aim of this study was to evaluate whether an energy restricted diet during lactation could program ovarian angiogenesis and growth factor and gonadotropin receptor expression.

Materials and Methods

Animals

The handling of the animals and the design of the study were approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro. Wistar rats were kept in a room with a controlled temperature (25 ± 1ºC) and an artificial dark-light cycle. After parturition, six Wistar rats with six pups each were separated into two groups: the control group, with free access to a standard laboratory diet containing (in grams per 100 g) 23 protein, 66 carbohydrate, 11 fat and 17,038.7 kJ/kg total energy; and the energy-restricted group, receiving the same standard laboratory diet in restricted quantities equaling60% of the amount consumed by the control group.

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Feed restriction

Feed restriction for treated rats started at birth and ended at weaning, when female pups from the same treatment group were housed in groups of three animals per cage and given unlimited access to food and water until 90 days of age. Afterwards, only the animals in the proestrus stage were sacrificed with a lethal dose of pentobarbital. Food consumption, body weight and linear growth were analyzed throughout the whole experimental period. At sacrifice, blood was collected for further evaluation of serum estradiol serum levels. Ovaries were excised and dissected. One ovary was kept at -80ºC for RT-PCR analysis while the other one was paraffin embedded, sectioned at 5-µm thickness and processed by routine histological analysis for evaluation of vessel density.

Semi-quantitative RT-PCR

All RNA samples were rid of contaminating DNA by using DNAse-free reagents (Invitrogen) according to the manufacturer's protocol. Then, 1 µg of RNA sample was used in a 20 µl cDNA reaction using Oligo dT and the Superscript III cDNA synthesis system (Invitrogen) according to the manufacturer's protocol. PCRs were prepared using the equivalent of 2 µl of cDNA per 50 µl reaction (triplicate) for each respective primer set using PCR reagents and Platinum Taq polymerase (Invitrogen). The applied PCR primers and the cycle profiles used are listed in Table 1. The optimal number of amplification cycles for each gene was determined in order to guarantee that the product levels were analyzed in the linear phase of amplification. All amplified cDNA fragments were run on a 1.5% agarose gel stained with ethidium bromide, visualized under UV transillumination and analyzed with the Image J software. In addition, to provide an appropriate internal control, co-amplification of a 450-bp fragment of GAPDH mRNA was carried out in each sample.

Vessel density

Paraffin sections of 5 µm from the left ovary of five animals from each group were taken at intervals of 50 µm and mounted on slides. The total number of sections analyzed was 15-20 per ovary. Routine hematoxylin-eosin staining was performed for histological examination under a light microscope. All vessels in the sections of the whole follicular internal theca were counted under 400X magnification.

Hormone assay

Estradiol serum concentrations were determined by a specific radioimmunoassay (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). The intra- and inter-assay variation coefficients for E2 were 6.4 and 5.9%, respectively, and the sensitivity of the radioimmunoassay was 0.8 pg/ml.

Statistical analysis

Statistical analysis was performed by Student's t-test. All results are shown as mean ± SEM. $P < 0.05$ was considered statistically significant.

Table 1. Oligonucleotide sequences used for amplification of reverse transcriptase-polymerase chain reactions and cycling conditions for the different sets of pairs.

Gene	Sequence (5' – 3')	Cycle profile	Cycles
bFGF	gaaccggtacctggctatga ccgttttggatccgagttta	94ºC/2min, 94ºC/1min, 55ºC/1min, 72ºC/1min	35
VEGF	gcccatgaagtggtgaagtt actccagggcttcatcatt	94ºC/2min, 94ºC/1min, 55ºC/1min, 72ºC/1min	33
FGFR	ctctgtggtgccttctgaaca ttcacctcgatgtcttcag	94ºC/2min, 94ºC/1min, 55ºC/1min, 72ºC/1min	33
KDR	ccaagctcagcacacaaaaa ccaaccactctgggaactgt	94ºC/2min, 94ºC/1min, 55ºC/1min, 72ºC/1min	32
Flt-1	tttatcagcgtgaagcatcg ccgaatagcgagcagatttc	94ºC/2min, 94ºC/1min, 55ºC/1min, 72ºC/1min	32
FSHR	ctcatcaagcgacaccaaga ggaaaggattggcacaagaa	94ºC/2min, 94ºC/1min, 55ºC/1min, 72ºC/1min	36
LHR	atggccatcctcatcttcac tggattggcacaagaattga	94ºC/2min, 94ºC/1min, 55ºC/1min, 72ºC/1min	33
GAPDH	accacagtccatgccatcac tccaccaccctgttgctgta	94ºC/3min, 94ºC/30s, 58ºC/2min, 72ºC/2min	30

bFGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor; FGFR: FGF receptor; KDR: kinase-insert domain receptor; Flt-1: Fms-like tyrosine kinase-1; FSHR: follicle-stimulating hormone receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; LHR: luteinizing hormone receptor.

Results

Food consumption, body weight and linear growth are shown (Fig. 1). The ER group had a decrease ($P < 0.007$) in food consumption compared to the C group after day 77 until the end of experiment. There was a decrease ($P < 0.0001$) in the ER group body weight from day 10 to day 65. The ER group also had a decrease ($P < 0.003$) in linear growth from day 11 to day 45.

Vessel density, considering arteries, capillaries and veins counted inside the theca layer of preantral and antral follicles was not changed in the ER group (C = 17.26 ± 2.306; ER = 17.14 ± 1.576). Similar findings were observed for systemic estradiol (C = 125.4 ± 20.4 pg/ml; ER = 102.1 ± 9.3 pg/ml) concentrations.

An energy restricted diet during lactation resulted in a significant increase in ovarian mRNA expression for bFGF and VEGF. However, their receptors were not altered. Receptor expression for FSH and LH (Fig. 2) were reduced ($P < 0.001$) after diet treatment.

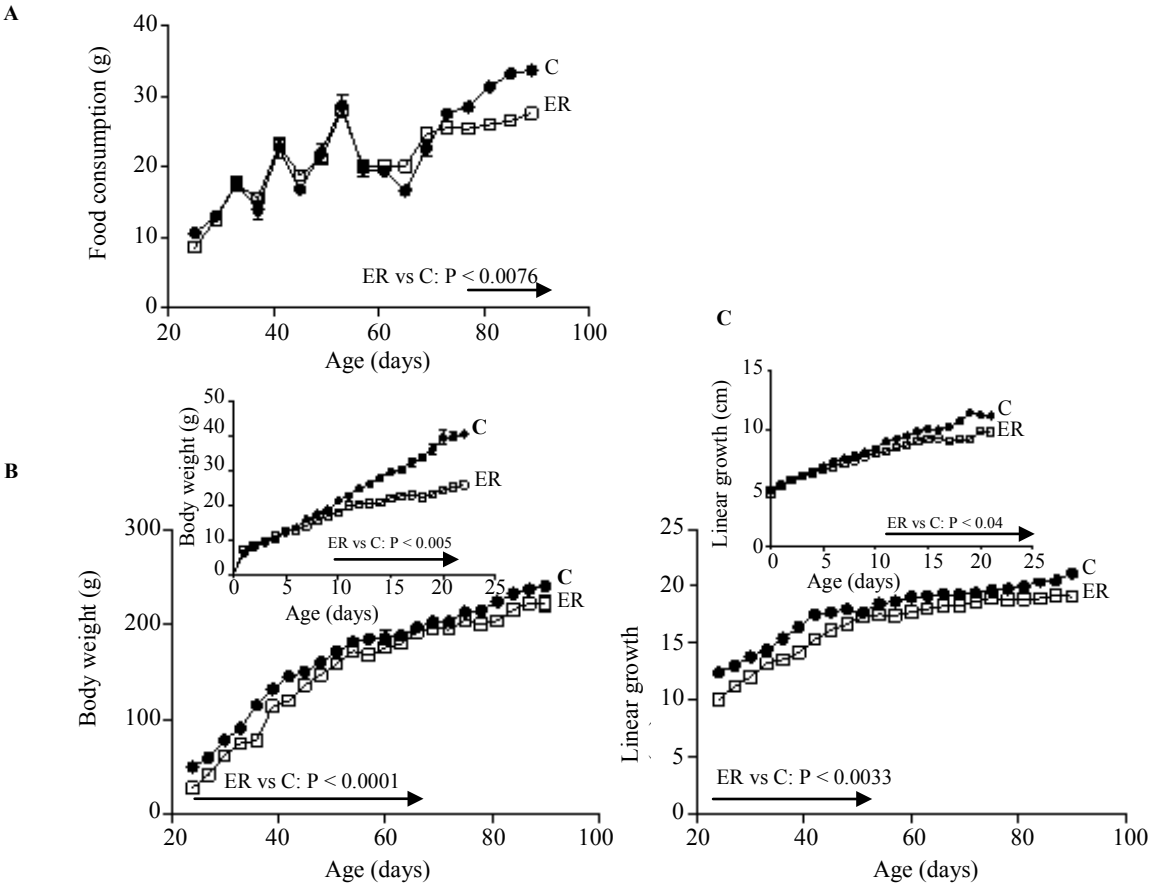

Figure 1. Food consumption (panel A), body weight (panel B) and linear growth (panel C) in the control (C) and energy restricted (ER) groups. Values are given as mean ± SEM of 14 animals per group.

Discussion

This study is in agreement with others that provide further evidence that early malnutrition can program the function of several systems, showing that the quantity and quality of nutrition during the perinatal period generates consequences in adulthood (Waterland and Garza, 1999; Armitage *et al*., 2005; Guzman *et al*., 2006).

In regard to food consumption, there was no difference between the groups until day 80. After this, there was a significant decrease in the ER group until the end of the experiment. Body weight and linear growth were reduced from the first days of life until puberty (around 40 days of age), probably due to maternal energy malnutrition during lactation. We can assume that the normal food consumption after weaning (21 days of age) could be responsible for the catch up of body weight and linear growth. The reduction in food intake in the ER group after day 80 could lead to a later alteration in body weight. Our results corroborate previous findings showing that malnutrition during the perinatal period is associated with a reduction in growth rate (Guzman *et al*., 2006; Zambrano *et al*., 2006).

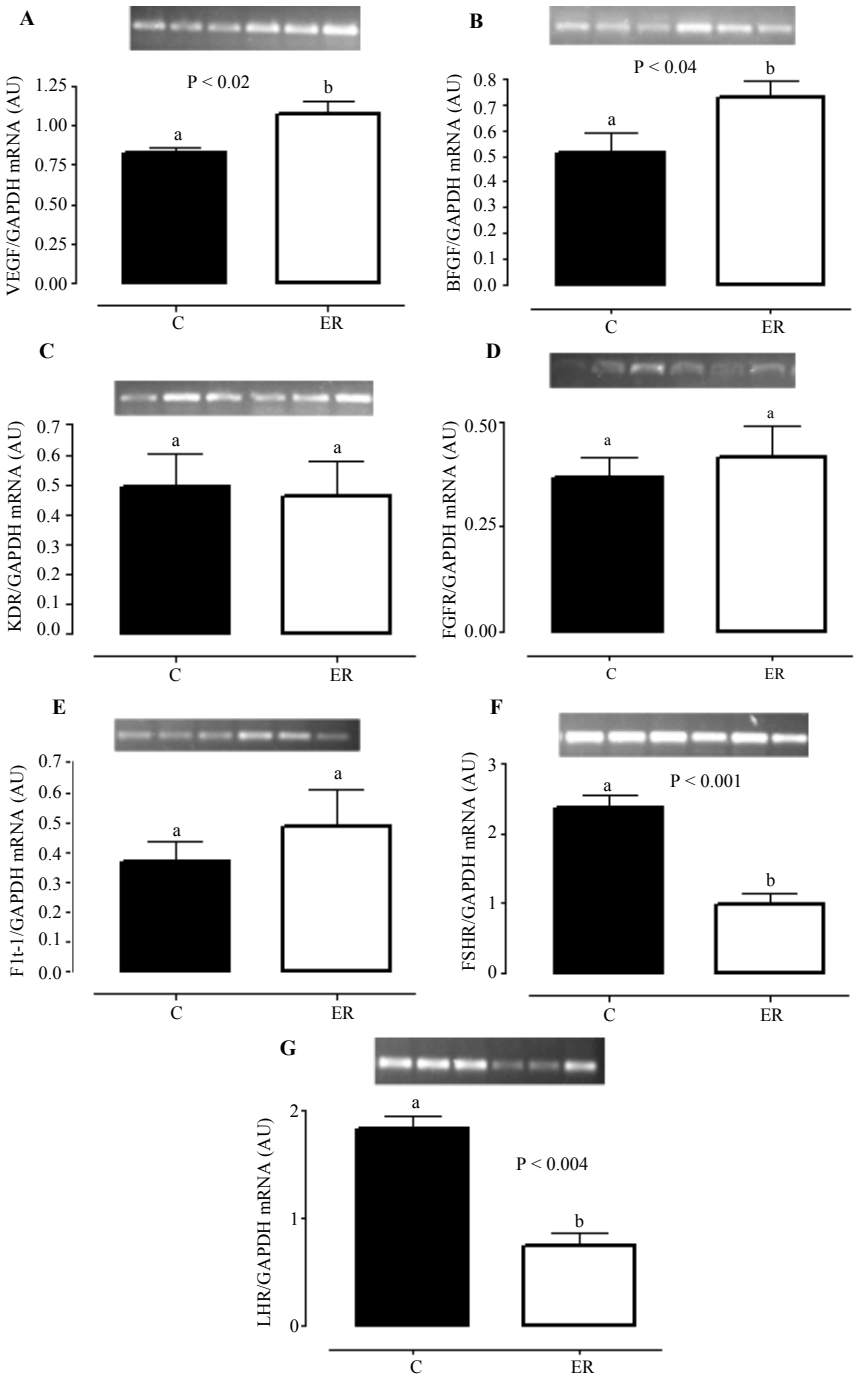

Figure 2. Expression of VEGF (A), bFGF (B), KDR (C), FGFR (D), Flt1 (E), FSHR (F) and LHR (G) genes in the ovaries of the control (C) and energy restricted (ER) groups. After RT-PCR reactions, the amplified fragments were run on a 1.5% agarose gel and visualized by UV transillumination. A representative ethidium bromide-stained gel depicting products for the expression of the different genes is shown above each graph. The ratios between the signal intensities (arbitrary units) of VEGF, bFGF, KDR, FGFR, Flt-1, FSHR and LHR are represented as means ± SEM of five animals per group. Different letters indicate a statistical significance.

Female fertility depends on follicular development with adequate maturation of primordial follicles to the mature stage of the Graafian follicle. We have recently shown that both protein-energy and energy malnutrition cause a significant decrease in folliculogenesis (Cavalcante *et al*., 2009). FSH is undoubtedly the primary stimulus for cyclic recruitment (McGee *et al*., 1997). LH is also important in promoting

major changes in ovarian preovulatory follicles, including terminal differentiation of follicular cells and oocyte maturation. These events are required for ovulation of a fertilizable egg (Hizaki *et al*., 1999). The low expression of FSHR and LHR in the ovary of rats whose mothers were submitted to an energy restricted diet during lactation could explain the decrease in the folliculogenesis process previously mentioned. Indeed, herein we demonstrated that there is a low expression of FSHR and LHR, reinforcing the notion that the decreased ovarian response to these hormones could be the reason for the alterations in folliculogenesis already described for this experimental model (Cavalcante *et al*., 2009). These results are very similar to those seen in animals whose mothers were submitted to protein-energy malnutrition during lactation (Faria *et al*., 2010).

Despite the reduction in FSHR and LHR expression, and the previous alteration in folliculogenesis (Cavalcante *et al*., 2009), there was no alteration in estradiol serum concentration. We could hypothesize that estradiol synthesis in sites outside the ovary, such as liver or adipose tissue, would have kept the hormone levels normal.

Angiogenesis plays an important role in follicular development. The capillary network in dominant follicles is more extensive and more permeable than in other follicles (Reynolds, 1973), increasing hormone and growth factor uptake (McNatty *et al*., 1981). The capillary network, limited to the thecal cell layer during follicular development, is stimulated by angiogenic factors (Koos *et al*., 1993). VEGF and FGF have been characterized as potent promoters of angiogenesis (Redmer and Reynolds, 1996). VEGF may also have direct mitogenic effects on granulosa cells in vitro and could directly stimulate follicle growth in the rat ovary (Otani *et al*., 1999). It has been reported that bFGF is able to induce primordial follicles to initiate development (Nilsson *et al*., 2001). Despite the fact that both bFGF and VEGF are stimulated by FSH (Christenson and Stouffer, 1997), in the current experiment we have shown that the gene expression of both factors was increased while FSHR expression was decreased. These results are not in agreement with our previous report that maternal protein-energy malnutrition during lactation led to a decrease in the expression of KDR, Flt-1 and FGFR, while there was no alteration in the angiogenic factors bFGF and VEGF (Ferreira *et al*., 2010). The gene expression of FSHR and LHR was reduced in these animals (Faria *et al*., 2010).

Those data suggest that other stimuli besides FSH could be important to regulate the expression of angiogenic factors and their receptors. We hypothesize that an increase in the expression of angiogenic factors could have been a compensatory mechanism to maintain the vessel density of follicles and provide enough hormones and other factors essential to folliculogenesis.

Although the results presented here cannot clarify the exact mechanisms by which an energy restricted diet programs folliculogenesis, they surely suggest that the lower ovarian response to FSH and LH and the alteration in the expression of angiogenic factors could be an important part of it.

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