Compensatory nutrition-directed mammary cell proliferation and lactation in rats

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The proper use of a time-dependent and controlled nutrition regimen during the hormonesensitive growth phase before first parturition can significantly affect mammary growth and subsequent lactation performance. The objective of the present study was to determine if a compensatory nutrition regimen improves lactation performance by affecting proliferation and apoptosis of mammary epithelial cells. Forty female rats (7 weeks of age, average weight 148 g) were assigned to either (1) control, free access to diet or (2) stair-step compensatory nutrition regimen, an alternating 3-4-week schedule beginning with an energy-restricted diet (31.2 % restriction) for 3 weeks, followed by the control diet for 4 weeks. Estimated milk yield was greater (P < 0.05) on day 15 of lactation in the compensatory nutrition group than in the control group. Mammary cell proliferation values were 1.4- and 2.7-fold greater in mammary tissue from the compensatory group during pregnant and early lactating stages respectively, compared with those from the control group. Ornithine decarboxylase (EC 4.1.1.17) mRNA was 24 % higher (P < 0.05) in mammary tissues of rats from the compensatory nutrition group during pregnancy than in those from the control group. These results indicate that the compensatory nutrition regimen imposed during the peripubertal growth phase stimulated mammary epithelial cell proliferation and improved lactation performance.

Compensatory growth: Lactation: Mammary tissue

Dietary restriction has a profound influence on the biology and health of animals including retardation of ageing and reduction of cancer incidence (Weindruch & Walford, 1988). While imposition of excessive dietary restriction on growing animals may inhibit normal development of the mammary gland, a well-controlled dietary energy restriction regimen positively effects mammary growth, development and subsequent lactation in rats (Park et al. 1988) and bovine species (Park et al. 1989; Peri et al. 1993; Barash et al. 1994; Choi et al. 1997). The stair-step (phased) compensatory nutrition regimen proposed and studied in our laboratory is designed so that development of mammary tissues is minimal during an energy restriction phase, while a compensatory growth phase immediately following energy restriction stimulates rapid and fuller development of the mammary gland. Studies from our laboratory (Park et al. 1988, 1989, 1997), as well as from others (Barash et al. 1994; Choi et al. 1997), show that lactation performance is increased by 8-17 % in animals reared on a compensatory nutrition regimen.

The cellular mechanisms responsible for the effects of nutritionally-induced compensatory growth on development, differentiation and regression of the mammary gland are not well studied despite the potentially beneficial role of a controlled nutrition regimen in improving lactation. A better understanding of how cell number is controlled may lead to strategies for prolonging lactation, not only by increasing peak yield, but also by reducing net loss of mammary cells. The objective of the present study was to determine if a compensatory nutrition regimen enhanced lactation performance by affecting proliferation and apoptosis of mammary epithelial cells in female rats.

Materials and methods

Animals and diets

Forty female Sprague–Dawley rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA) at 6 weeks of age. Rats were housed individually in metal wire cages.

Abbreviations: BrdU, bromodeoxyuridine; ODC, ornithine decarboxylase; SSPE, sodium chloride-sodium dihydrogen phosphate-EDTA disodium salt; TdT, terminal deoxynucleotidyl transferase.

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mesh bottomed cages and kept in an isolated room with controlled environment: 25°C and 50% relative humidity with 12 h light–dark cycle and free access to water. All experimental methods and procedures were approved by the University Animal Care and Use Committee.

Rats were acclimated to our facilities for a 1-week period. During this acclimation period, all rats were given *ad libitum* access to the control diet (modified AIN-76 diet; American Institute of Nutrition, 1977). At 7 weeks of age, rats weighing 120–157 g were randomly assigned to either control (*n* = 20, 148 ± 4 (SE 3.8) g) or stair-step compensatory nutrition regimen (*n* = 20, 147 ± 8 (SE 4.3) g) groups. The control group was offered free access to the control diet throughout the trial period; feed intake was recorded twice weekly and average daily intake was estimated. The compensatory nutrition group was subjected to a 3- and 4-week phased schedule beginning with an energy-restricted diet (31.2% energy restriction) for 4 weeks. The treatment diet during the energy restriction period was formulated to provide similar intakes of protein, vitamins, and minerals as the control diet but less energy (Table 1). During the energy restriction phase, treatment rats were fed on the energy-restricted diet at 60% of the average *ad libitum* intake of the control group: e.g. if the control group consumed 10 g/d, the amount of feed offered to the restricted group was 6 g/day. Thus, the overall energy intake of the compensatory nutrition rats was intended to be 13-4% less than that of the control group.

On completion of the nutrition regimen (approximately 14 weeks of age), rats were maintained on the control diet with free access for the remaining experimental period. All rats were mated during the second week of re-alimentation (11 weeks of age). The six rats that failed to conceive during the 10 d mating period were removed from the experiment leaving seventeen pregnant rats in each group.

### Table 1. Ingredients and chemical composition of experimental diets (g/kg)

<table>
<thead>
<tr>
<th>Ingredient*</th>
<th>Control</th>
<th>Energy-restricted†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>334</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>500</td>
<td>285</td>
</tr>
<tr>
<td>Maize starch</td>
<td>150</td>
<td>85</td>
</tr>
<tr>
<td>Cellulose (Alphacel)</td>
<td>50</td>
<td>85-3</td>
</tr>
<tr>
<td>Maize oil</td>
<td>50</td>
<td>129</td>
</tr>
<tr>
<td>Mineral mix (AIN 76)‡</td>
<td>35</td>
<td>58.5</td>
</tr>
<tr>
<td>Vitamin mix (AIN 76)‡</td>
<td>10</td>
<td>16.7</td>
</tr>
<tr>
<td>Choline bitratrate</td>
<td>2</td>
<td>3-3</td>
</tr>
</tbody>
</table>

**Chemical analysis**

| Crude protein | 180 | 314 |
| Gross energy (MJ/kg) | 18.9 | 21.7 |
| Digestible energy (MJ/kg)§ | 17.0 | 19.5 |

* All ingredients were purchased from ICN Biochemicals (Costa Mesa, CA, USA).
† Diet of the compensatory nutrition group during the energy restriction phase.
§ Digestible energy = 0.9 × gross energy (Nelson et al. 1974).

Litter size in both groups was adjusted to eight pups per dam on day 3 of lactation. Weights of dams and litters were measured every 2 d from day 3 to day 15 of lactation. Milk yield on day 15 of lactation was determined by the stepwise forward multiple regression equation from Sampson & Jansen (1984).

### Immunohistochemistry

Five randomly selected rats per treatment group were injected intraperitoneally with bromodeoxyuridine (Brdu, a thymidine analogue; 5 mg Brdu/kg body weight; Aldrich, Milwaukee, WI, USA) during mid-pregnancy (day 11), and early lactation (day 3) (five control (mean litter size 10.5 (SE 0.4); litter weight 67.3 (SE 2.4) g) and five compensatory nutrition rats (mean litter size 10.7 (SE 0.5); litter weight 68.0 (SE 2.0) g)). At 2 h after the Brdu injection the animals were killed using CO2. Inguinal mammary tissue was removed and fixed in Carnoy’s solution for 4 h at room temperature. Samples of mammary tissues from pregnant rats were also frozen rapidly in liquid N2, and stored at −70°C for subsequent RNA extraction and Northern blot analysis. Paraffin-embedded mammary tissues were sectioned at 5 μm and mounted onto glass slides using standard histological techniques. After rehydration, tissue sections were incubated in 2 M-HCl for 30 min to denature the DNA. The sections were blocked with normal horse serum and then treated with mouse monoclonal anti-BrdU (Boehringer-Mannheim, Indianapolis, IN, USA) for 1 h at room temperature. After washing with phosphate-buffered saline (50 mM-sodium phosphate, pH 7.4, 200 mM-NaCl), the sections were incubated with a biotin-conjugated goat antimouse immunoglobulin G (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Horseradish peroxidase complex reagents (Vector Laboratories) were used to localize the secondary antibody. The 3,3'-diaminobenzidine substrate was used in the colour reaction. More than 500 cells were counted in randomly selected areas of three tissue sections from each dam to measure the DNA synthesis labelling index. The labelling index was defined as the ratio BrdU-labelled cells: total number of cells counted, and was expressed as a percentage.

### Apoptosis in situ by end-labelling

Five randomly selected rats from each dietary group were killed during late lactation (day 20). Apoptotic mammary epithelial cells were essentially detected according to the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate–biotin nick end labelling (TUNEL) procedure described by Gavrieli *et al.* (1992). Briefly, paraffin-embedded tissue was sectioned at 5 μm and adhered to glass slides. The sections were deparaffinized and rehydrated for *in situ* specific labelling to fragmented DNA. Nuclei of tissue sections were stripped of proteins by incubation with 20 mg/l proteinase K (EC 3.4.21.64) for 30 min. Endogenous peroxidase was inactivated with 30 ml/l H2O2–methanol (1:49, v/v) for 30 min. After rinsing with distilled water, the sections were...
immerged in a buffer (30 mM-Tris, pH 7.2, 140 mM-sodium cacodylate, 1 mM-CoCl₂) containing TdT (0-3 units/μl) and 1 mm-biotinylated dUTP (Clontech, Palo Alto, CA, USA) and then incubated in a humid atmosphere at 37° for 60 min. On termination of the reaction the sections were treated with the avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories). A 3,3’-diaminobenzidine substrate was used in the colour reaction. Rat intestinal sections were used as the positive control. The apoptotic cell labelling index was calculated after counting more than 500 nuclei in randomly selected areas of three tissue sections from each dam. The labelling index was defined as the ratio positive nuclei: total number of nuclei counted, and was expressed as a percentage.

RNA extraction and dot and Northern blot analyses

Total RNA was extracted from mammary tissue of pregnant rats (day 11) by the modified Chomczynski & Sacchi (1987) method. Briefly, the mammary tissues were pulverized in liquid N2 and homogenized in lysis buffer containing 4M-guanidium thiocyanate, 25 mM-sodium citrate, 5 ml/l sarcosyl, 0.1 M-2-mercaptoethanol. The homogenate was extracted with phenol and chloroform. Total RNA in the aqueous phase was precipitated with isopropanol and sodium acetate, pH 4-2, and dissolved in diethyl pyrocarbonate-treated distilled deionized water. For dot blotting, total RNA from individual mammary tissues was electrophoresed on agarose gels (12 g/l) containing 2.2 M-formaldehyde and transferred to nylon membranes as previously described (Baik et al. 1992). For Northern blotting, poly(A)+ RNA was isolated from pooled total RNA using oligo deoxythymidine cellulose columns (5 Prime→3 Prime Inc., Boulder, CO, USA). Poly(A)+ RNA (6 μg per lane) was fractionated by electrophoresis on agarose gels (12 g/l) containing 2-2 M-formaldehyde and transferred to nylon membranes.

The membranes were baked for 30 minutes at 80° and prehybridized for 3 h at 42° in hybridization buffer containing 500 g/l formamide, 6X Denhardt’s solution and 2 ml/l SDS. The rat ornithine decarboxylase (EC 4.1.1.17; ODC) cDNA was donated by Dr P.J. Blackshear (Duke University, Durham, NC, USA). The γ-actin cDNA was obtained from Dr L.H. Kedes (University of Southern California, Los Angeles, CA, USA). The ODC cDNA insert was excised with the restriction enzyme EcoRI, and the γ-actin cDNA insert was excised with the restriction enzyme BamHI for subsequent labelling. The denatured cDNA probes were labelled with β²PdATP by random priming method (Multiprime DNA Labeling Systems, Amersham Life Science, Arlington Heights, IL, USA). Hybridization was performed for 17 h at 42°. The membranes were washed twice at room temperature in a solution containing 5X sodium chloride–sodium dihydrogen phosphate–EDTA disodium salt (SSPE) and 5 ml/l SDS, followed by washing twice at 37° in a solution containing 1X SSPE and 10 ml/l SDS. The membranes were exposed to X-ray film (Kodak, Rochester, NY, USA) with an intensifying screen at 70° for 72 h. The relative intensities of the autoradiograms were quantitated by laser densitometry (Personal Densitometer SI System, Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

All statistical analyses were performed with SAS/STAT version 6.11, (Statistical Analysis Systems Institute, Cary, NC, USA). Data on food intake and litter weight were analysed using one-way ANOVA with repeated measures. Gene expression and lactation data were analysed with the independent Student’s t test for unpaired observations. Pearson’s correlation coefficients were used to assess the relationship between selected variables. Differences were considered significant at \( P < 0.05 \).

Results and discussion

Mammary growth is the major determinant of milk yield capacity and persistency of lactation (Forsyth, 1996). Growth and differentiation of the mammary gland are regulated by various hormones, growth factors, and nutritional factors including the plane of nutrition (Borello & Oka, 1989; Imagawa et al. 1990). Several studies have shown that a controlled nutrition regimen imposed during early developmental stages before first parturition can influence significantly mammary growth, differentiation and subsequent lactation performance (Park et al. 1988, 1989, 1997; Peri et al. 1993; Choi et al. 1997). To develop a better understanding of nutritionally-mediated mammary development and lactation, our laboratory has proposed and studied a step compensation nutrition regimen. This nutrition regimen, a combination of both energy restriction and re-alimentation, was designed primarily to improve lactation potential by exploiting the characteristics of the compensatory growth phenomenon during peripubertal developmental stages (Park et al. 1988, 1989, 1994a, 1997; Choi et al. 1997).

The theoretical energy intakes of the compensatory nutrition group were 68.8% and 100% for energy restriction and re-alimentation phases respectively, with an overall 13.4% reduction in energy intake compared with the control group. Actual total energy intakes (Table 2) of the compensatory nutrition regimen rats were 68.3% of the intake of the control rats during the energy restriction phase and 104.2% of the intake of the control rats during the re-alimentation period. This corresponds to an approximate overall 11% reduction in energy intake, indicating that energy intakes were regulated as intended by design. Results from earlier studies show consistently that the compensatory nutrition regimen improves efficiency of energy (weight gain/energy intake) of all animal species examined (Park et al. 1987, 1994a; Choi et al. 1997).

Overall lactation performance was improved in the compensatory nutrition regimen group. Average litter weight and milk yield per dam are shown in Table 3. While the initial litter weight and size were not different between groups, the average litter weight on day 15 of lactation was 23% higher \( (P < 0.05) \) in the compensatory nutrition group than in the control group. Dams reared on the compensatory nutrition regimen produced 17% more \( (P < 0.05) \) milk than did dams on the control diet. Lactation was 23% higher \( (P < 0.05) \) in the compensatory nutrition regimen group. Average litter weight and milk yield per dam are shown in Table 3.
Table 2. Total energy intake of rats on experimental diets (MJ)†
(Mean values with their standard errors; ranges in parentheses)

<table>
<thead>
<tr>
<th>Nutrition regimen</th>
<th>Control</th>
<th>Compensatory nutrition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (Range)</td>
<td>SEM</td>
</tr>
<tr>
<td>Energy restriction</td>
<td>5.62 (4.82-6.39)</td>
<td>0.08</td>
</tr>
<tr>
<td>(7-10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Re-alimentation</td>
<td>7.06 (6.52-7.35)</td>
<td>0.04</td>
</tr>
<tr>
<td>(10-14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>12.68 (11.06-13.94)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those of the control group, * P<0.05.
† Each value represents total energy intake per rat during the indicated period. Total energy intake (MJ) = total feed intake (kg) x digestible energy of experimental diet (MJ/kg).
§ For details of the nutrition phases, see pp. 177-178.
§ Three rats from each group, which failed to conceive, were removed from the experiment during the re-alimentation phase.

The increase in lactation performance in the compensatory nutrition group was probably due to the compensatory mammary growth that occurred during the re-alimentation phase; this period encompassed puberty through to late gestation.

Mammary cell proliferation was determined by the extent of BrdU incorporation into DNA (Table 4). Cell proliferation was unchanged between pregnant and early lactating stages in mammary tissues from the compensatory nutrition rats; however, mammary cell proliferation decreased 50% (P<0.05) from pregnancy to early lactation in tissues of control rats (5.8 v. 2.9). This postpartum decrease in proliferative activity was unexpected since peak cellular proliferation in rat mammary tissues occurs during early gestation and continues through early lactation (Joshi et al. 1986). Mammary cell proliferation rates were 1-4- and 2-7-fold greater in mammary tissue from the compensatory group during pregnant (P<0.05) and early lactating (P<0.05) stages respectively, compared with those from the control group (Table 4). It is possible that accelerated compensatory growth induced during re-alimentation may alter the level and activities of transcription factors regulating cell proliferation through changes in the hormonal status of the animal. There were no significant correlations between mammary cell proliferation and initial litter size and litter weight in either experimental group.

Table 3. Litter weight and milk yield in rats fed on a compensatory nutrition regimen compared with controls
(Mean values with their standard errors; ranges in parentheses)

<table>
<thead>
<tr>
<th>Nutrition regimen</th>
<th>Control</th>
<th>Compensatory nutrition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (Range)</td>
<td>SEM</td>
</tr>
<tr>
<td>Initial litter size</td>
<td>10.9 (7-15)</td>
<td>0.6</td>
</tr>
<tr>
<td>Litter weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>68.7 (47.1-87.4)</td>
<td>4.0</td>
</tr>
<tr>
<td>On day 3 of lactation‡</td>
<td>66.7 (53.7-90.2)</td>
<td>2.6</td>
</tr>
<tr>
<td>On day 15 of lactation‡</td>
<td>162.2 (125.2-195.0)</td>
<td>11.1</td>
</tr>
<tr>
<td>Milk yield (g)§</td>
<td>19.6 (14.5-25.6)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those of the control group, * P<0.05.
† Number of dams.
‡ Adjusted to eight pups per dam on day 3.
§ Milk yield per litter (g): Weight is average pup weight on day 15 of lactation (g); gain is average rate of pup weight gain on day 15 (g/d); and 8 is litter size on day 15 of lactation.

For details of the nutrition phases, see pp. 177-178.
Nutritionally-directed mammary growth

Table 4. Labelling index of rat mammary cells exhibiting cell proliferation and apoptosis during pregnancy and lactation (%)‡

<table>
<thead>
<tr>
<th>Nutrition regimen</th>
<th>Control</th>
<th>Compensatory nutrition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>Mid pregnancy</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Early lactation</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Late lactation</td>
<td>5</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those of the control group, * P < 0.05.
Mean value was significantly different from the early lactation value, ‡P < 0.05.

† For details of cell proliferation and apoptotic cell identification procedures, see pp. 178-179.

Growth hormone and prolactin are associated with the regulation of the expression of ODC (Rillema et al. 1981). The expression of ODC is also sensitive to dietary energy modifications (Maudsley et al. 1976; Baik et al. 1992; Yoshinaga et al. 1993; Park et al. 1994b). ODC, the rate-limiting enzyme in polyamine biosynthesis, is closely correlated with cell proliferation and differentiation (Pegg & McCann, 1982). The level of ODC mRNA was 24% greater (P < 0.05) in tissues from the compensatory nutrition group than in those from the control group on day 11 of pregnancy (Fig. 1). The expression of ODC may be triggered by elevated levels of growth hormone induced during compensatory growth, thereby affecting the rate of polyamine biosynthesis, and subsequently the synthesis of milk protein in the mammary gland (Oka et al. 1982).

In addition to cell proliferation, lactation performance may also be affected by apoptosis. Apoptosis is a mode of physiological cell death that occurs naturally in rodent mammary tissue during involution (Walker et al. 1989). Milk output decreases gradually, probably because mammary alveolar secretory cells are lost and synthetic activity per cell declines (Knight & Wilde, 1993; Park & Jacobson, 1993). The inhibition of apoptosis might prolong secretory cell longevity (Strange et al. 1992; Li et al. 1996).

Apoptosis was determined by specific labelling of DNA breaks in situ (Table 4). While apoptotic mammary cells were detectable in mammary tissues from both groups during late lactation (20d), there was no significant (P > 0.05) difference between the two dietary treatments. There was also no significant correlation between apoptotic cell numbers and litter size or litter weight.

Conclusions

The compensatory nutrition regimen imposed during the peripubertal growth phase stimulated the expression of ODC, a gene regulating mammary cell proliferation. Rats on the compensatory nutrition regimen had increased cell proliferation and enhanced lactation performance.

In addition to improving lactation performance, our compensatory nutrition regimen may offer opportunities in...
the development of breast cancer prevention strategies for young adolescent women. DeWard & Trichopoulos (1988) hypothesized that breast cancers are most likely to occur between the onset of puberty and the birth of the first baby. Mammary tissue is sensitive to cancer induction during peripubertal development because of the high degree of cell proliferation. On the other hand, the stimulation of rapid growth and fuller differentiation of parenchyma tissue of mammary gland during gestation inhibits tumourigenesis (Russo & Russo, 1986, 1995). The extent of the mammary epithelial cell proliferation and differentiation depends largely on nutritional status (De-Ward & Trichopoulos, 1988; Park & Jacobson, 1993). Our compensatory nutrition regimen can not only enhance the degree of mammary differentiation, but can curtail the span of time between the onset of mammary development and fully differentiated mammary tissues. Thus, the critical mammary growth period sensitive to cancer induction may be shortened.

Acknowledgements
The authors thank Julie Berg for secretarial assistance. This research was supported in part by a grant (92-37208-8122) from the US Department of Agriculture.

References
Barash H, Bar-Meir Y & Bruckental I (1994) Effect of low-energy diet followed by a compensatory diet on growth, puberty and pregnancy of young adolescent women. DeWard & Jacobson, 1993). Our compensatory nutrition regimen can not only enhance the degree of mammary differentiation, but can curtail the span of time between the onset of mammary development and fully differentiated mammary tissues. Thus, the critical mammary growth period sensitive to cancer induction may be shortened.

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References


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