Effects of Body Weight Gain Reduction Resulting From Chemopreventive Agent Treatment on Mammary Gland Morphology

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Abstract: Moderate reductions (≤15%) in body weight gain, similar to those observed after administration of some chemopreventive agents in chemically induced mammary cancer models, will result in decreased mammary cancers (up to 55%). The objective of this study was to determine whether changes in mammary gland differentiation, proliferation, apoptosis, and estradiol and progesterone levels are affected by moderate reductions in body weight induced after chemopreventive agent treatment and dietary restriction. The body weights of female Sprague-Dawley rats were reduced by dietary restrictions to match those of rats receiving 4-hydroxyphenylretinamide (4-HPR) at a dose known to inhibit methylNitrosourea (MNU)-induced mammary cancers. 4-HPR supplementation or dietary restrictions began 1 wk before MNU administration at 50 days of age. Mammary gland differentiation, proliferation, apoptosis, and serum levels of estradiol and progesterone were measured at 50, 57, and 71 days of age. Casein expression, proliferating cellular nuclear antigen expression, and apoptosis were not significantly different from controls in the dietary-restricted group. Proliferating cellular nuclear antigen expression was significantly lower in 4-HPR-treated animals than in controls at 57 days of age. The diameter of the mammary gland ducts was smaller at 71 days of age in the treatment groups. A decrease in estradiol levels for each group was observed at 50 days of age, but not at later time points. Progesterone levels were reduced in the 4-HPR group, but not in the dietary-restricted group, during each time period. It would appear that the observed decrease in mammary cancers observed with moderate reductions in body weight gain might be due to multiple related factors different from those related to 4-HPR treatment.

Introduction

In the evaluation of potential chemopreventive agents, dose levels of a compound are selected such that no overt signs of toxicity will be observed. Until recently, a moderate depression in body weight gain of ≤15% in rodent models of mammary cancer was considered acceptable when the maximum tolerated dose of a compound was selected. However, we have observed that moderate reductions in body weight gain (≤15%) can significantly reduce the number of mammary cancers induced by a chemical carcinogen. Reductions in body weight gain of 3%, 6%, 9%, 12%, and 15%, initiated before methylNitrosourea (MNU) treatment by restrictions in food intake, reduced mammary cancer multiplicities by 14%, 15%, 41%, 44%, and 55%, respectively, compared with ad libitum-fed controls (1). We have also determined the effect of different patterns of reduction in body weight gain (acute vs. chronic) on cancer multiplicity in the MNU model of mammary cancer (2). For example, a significant decrease in mammary cancers (36%) occurred in rats that underwent a 12% acute reduction in body weight gain compared with ad libitum-fed controls. In contrast, chronic reductions in weight gain of 12% had minimal effects on cancer multiplicities (2). These findings have demonstrated that at least part of the chemopreventive activity of some agents may not be attributed to the agent per se but, rather, to the depressions in body weight gain resulting from administration of the compound.

Other investigators have also demonstrated that calorie restriction will significantly reduce the incidence and number of tumors in chemically induced cancer models (3–9). Most of these studies included dietary restrictions of 40–50% of ad libitum-fed controls. Some studies, however, did focus on moderate (<15%) dietary restrictions (6,7). Despite the vast amount of literature available on depressions in body weight and calorie restriction in relation to decreased carcinogenesis, the mechanism responsible for this relationship remains in question. Possible mechanisms that have been reported to explain the effect of dietary restrictions on carcinogenesis include decreased cellular proliferation (10), altered immune function (11), altered growth factor responsiveness (12), re-
duced insulin levels (13), suppression of prolactin and estrogen secretion (14), increased adrenal cortical activity (15–17), enhanced DNA repair (18), and increased activity of antioxidant enzymes (19). Thompson et al. (20) reported a dose-dependent increase of p27Kip-1 protein with calorie restriction, suggesting that calorie restriction inhibits carcinogenesis in part by delaying cell cycle progression. Birt et al. (21) demonstrated a reduction in protein kinase C activity with calorie restriction in a chemically induced skin cancer model.

The objective of the present study was to determine whether changes in various parameters (mammary gland differentiation, proliferation, apoptosis, and serum levels of estradiol and progesterone) are affected by moderate reductions in body weight gain induced by chemopreventive agent treatment and by dietary restriction. Furthermore, the purpose of this study was to determine whether changes induced by dietary restriction are the same as those that occur after 4-hydroxyphenylretinamide (4-HPR) treatment. Mammary gland differentiation, proliferation, apoptosis, and serum levels of estradiol and progesterone were measured during three time periods around the time that the carcinogen MNU is typically given (50 days of age). The groups included in this study were ad libitum-fed controls, 4-HPR-treated animals, and animals with body weights matched to the average body weight of 4-HPR-treated animals. 4-HPR was utilized in this study because it is a representative chemopreventive agent that suppresses MNU-induced mammary cancers and also induces depressions in body weight gain shortly after its administration.

Methods

Animals

Female Sprague-Dawley rats were obtained at 21 days of age from Harlan Sprague Dawley (Indianapolis, IN) virus-free colony number 202. The animals were provided Teklad (4%, diet 001) mash diet (Harlan Teklad, Madison, WI) and tap water ad libitum during the quarantine period. When the experiment was initiated, the rats were housed individually in polycarbonate cages. The rats were kept in a room artificially lighted for 12 h each day and maintained at 72 ± 2°F. At 1 wk before the dietary restriction experiment, the rats were randomly assigned to the control and experimental groups. The average mean body weight for each group was within ±3 g at the start of the study.

Study Design

The control animals (n = 30) were fed Teklad (4%) mash diet ad libitum. The experimental animals (n = 30/group) consisted of a group treated with 4-HPR (782 mg/kg of diet) and a group subjected to food restriction, such that the body weights of these animals were matched to the body weights of the 4-HPR-treated animals. Food consumption was measured daily in the control group and the animals treated with 4-HPR. Food restriction and 4-HPR treatment were initiated at 43 days of age. The amount of food provided to the food-restricted animals was adjusted daily. All rats were weighed daily.

The carcinogen MNU (Ash Stevens, Detroit, MI) was administered via the jugular vein (50 mg/kg body wt iv) at 50 days of age. Throughout the study period, vaginal smears were taken (n = 10 rats/group) to determine estrous cycle lengths.

At 50, 57, and 71 days of age, 10 rats from each group were sacrificed. Liver, adrenal gland, uterus, and ovary weights from each animal were obtained, and each tissue was histologically evaluated. Whole mounts of the mammary glands of five randomly selected rats per group within each time period were examined for possible changes in morphology. Serum levels of estradiol and progesterone were determined in 10 rats per group for each time period.

At sacrifice, mammary tissue was collected from each animal for measurements of differentiation, proliferation, and apoptosis. Inguinal/abdominal mammary gland pairs were excised from five rats in each group for each time period (50, 57, and 71 days of age). Northern blot analyses were performed on these tissues to determine the mRNA level of caspase-3 expression. Mammary tissue was removed from the linea alba of each rat, fixed in neutral-buffered formalin for 24 h, and processed to paraffin blocks. Immunohistochemistry was used to determine the expression of proliferating nuclear antigen (PCNA). Apoptosis was measured using the TdT-mediated dUTP nick end labeling technique.

Mammary Gland Whole Mount Preparations

Whole mounts of the mammary glands of five rats per group within each time period were examined for possible changes in morphology. Immediately after euthanasia, a midline incision was made, and the skin and mammary tissue were rapidly pulled away from the peritoneal wall in the abdominal/inguinal area. The mammary gland was dissected away from the skin, placed on a microscope slide, and fixed in formalin for 24 h. Each slide was placed in an absolute ethanol-glacial acetic acid (3:1) solution for 1 h. The slides were then placed in acitone for 2 h, in 70% ethanol for 15 min, and in distilled water for 5 min and stained with alum carmine overnight. Mammary glands were washed with 35%, 50%, 70%, 95%, and 100% ethanol, each in 20- to 30-min increments. The mammary glands were transferred to toluene for 60 min. Each mammary gland was removed from the slide, trimmed, and mounted on a new slide using coverslips and Permount. Each mammary gland was semiquantitatively evaluated by two investigators for degree of proliferation and differentiation by observing the size and number of terminal end buds, alveolar buds, and terminal ducts. To further quantitate possible changes in mammary gland structure, size, and morphology, a random field was selected and photographed at the same magnification from each whole mount.
of the mammary gland for each group at 71 days of age. On each photograph of the random fields, the diameter of the mammary gland duct was measured in 10 different areas. The number of terminal end buds, alveolar buds, and terminal ducts, as well as the diameter of each individual structure, were determined for each random field.

Hormone Analyses

Blood samples were obtained from 10 animals of each group per time period to measure serum levels of estradiol and progesterone. Animals were sacrificed using CO₂ gas, and blood was collected intravenously through the vena cava. Each blood sample was centrifuged at 4°C for ~15 min at 2,800 rpm. The serum was obtained from each sample and stored at ~80°C until radioimmunoassays were performed. Serum levels of estradiol were determined using a solid-phase 125I-double-antibody kit (Diagnostic Products, Los Angeles, CA). Progesterone was measured using a solid-phase 125I-radioimmunoassay Coat-a-Count kit (Diagnostic Products). Standards for each serum steroid were developed for the rat by following a method previously described by Chard (22).

Northern Blot Analysis of Casein Expression

Northern blot analyses were performed to determine the mRNA level of casein expression of inguinal/abdominal mammary gland pairs from five rats in each group for each time period. RNA was isolated as described in a protocol using the Trizol reagent (Life Technologies, Grand Island, NY) and is based on the work of Chomczynski and Sacchi (23). The concentration and purity of the RNA were spectrophotometrically determined by reading the absorbance at 260 and 280 nm.

Each sample was electrophoresed through a 1.2% agarose-formaldehyde gel and transferred onto a Nytran nylon membrane (Schleicher & Schuell, Keene, NH) using the TurboBlotter rapid downward capillary transfer system kit (Schleicher & Schuell). RNA was linked covalently to the membrane using a cross-linking apparatus (Fisher Scientific) and stained with methylene blue.

The blots were prehybridized in 5 ml of prehybridization buffer [1% sodium dodecyl sulfate (SDS), 5× Denhardt’s solution, 5× NaCl-sodium phosphate-EDTA (SSPE), 50% formamide, and 100 µg/ml salmon sperm DNA] for 1–2 h using a hybridization oven. A β-casein cDNA probe was labeled using a Prime-It II random primer labeling kit (Stratagene, La Jolla, CA). The β-casein cDNA probe was provided by Dr. Jeffrey Rosen (Baylor College of Medicine, Houston, TX). The probe was purified using a Probe Quant Sephadex G-50 Micro column, added to the hybridization tubes, and hybridized overnight at 42°C.

The membrane was washed twice for 5 min with 0.1× SSPE-0.1% SDS at 42°C, twice for 30 min each with 0.1× SSPE-0.1% SDS at 68°C, and twice for 5 min with 2× SSPE at room temperature. The membrane was placed in an autoradiograph cassette and exposed to double-emulsion film using a Biomax intensifying screen (Eastman Kodak, Rochester, NY) at –70°C for 48 h.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks of inguinal mammary tissue from 10 animals per group for each time period (50, 57, and 71 days of age) were used to perform immunohistochemistry to determine PCNA expression. Standard immunostaining procedures (24–26) were used to determine PCNA expression. Paraffin tissue sections (5 µm) were mounted on SuperFrost/Plus slides and heated at 58°C for 1 h to adhere the tissue sections onto the slides. Tissue sections were deparaffinized in three changes of xylene and subsequently rehydrated with one change of 100% ethanol, 95% ethanol, and 70% ethanol, each in 5-min increments. The sections were then placed in a Tris-buffered saline bath (0.05 M Tris base, 0.15 NaCl, 0.0002% Triton X-100, pH 7.6). Before immunostaining, high-temperature antigen recovery was performed as described previously (27,28), such that the tissue sections were boiled in citrate buffer (10 mM, pH 6.0) for 10.0 min, cooled at room temperature for 15 min, rinsed with deionized water, and transferred to Tris buffer.

Endogenous peroxidases were quenched using an aqueous solution of 3% H₂O₂ for 5 min. Goat serum (1%) was added for 20 min at room temperature to block nonspecific immunostaining. The monoclonal antibody to PCNA (Oncogene Science, Cambridge, MA) was added at 0.03 µg/ml for 1 h at room temperature. Negative controls were incubated with 1% goat serum for 1 h. Immunodetection was performed using a universal mouse kit (Biogenex, San Ramon, CA). Tissue sections were incubated at room temperature with biotinylated goat anti-mouse for 20 min. The sections were then incubated with peroxidase-labeled avidin for 20 min. A 3,3′-diaminobenzidine (DAB) tetrahydrochloride supersensitive substrate kit (Biogenex) was used to visualize the antibody-antigen complex. Tissue sections were rinsed in deionized water, lightly counterstained with hematoxylin, and dehydrated, and coverslips were attached with Permount.

Each slide was evaluated at ×400 such that random fields were selected to count 1,000 cells per tissue section. Labeling indexes were determined as follows: 100 × no. of cells that stained positive for PCNA ÷ total no. of cells counted.

Apoptosis

Formalin-fixed paraffin-embedded tissue blocks of inguinal mammary tissue from 10 animals per group for each time period (50, 57, and 71 days of age) were used to measure apoptosis by TdT-mediated dUTP nick end labeling. Paraffin sections (5 µm) of tissue were mounted on Superfrost/Plus slides and heated at 58°C for 1 h. Tissue sections were
deparaffinized in three changes of xylene and rehydrated with one change of 100% ethanol, 95% ethanol, and 70% ethanol, each in 5-min increments. Then the sections were placed in Tris-buffered saline (0.05 M Tris base, 0.15 M NaCl, 0.0002% Triton X-100, pH 7.6).

A TdT-FragEL DNA fragmentation detection kit (Onco-gene Research Products, Cambridge, MA) was used to stain apoptotic nuclei. Proteinase K (1:100 dilution in 10 mM Tris, pH 8) was added to the tissue specimens and incubated at room temperature for 20 min. Endogenous peroxidases were quenched with an aqueous solution of 3% H₂O₂ for 5 min. Sections were treated with a TdT equilibration buffer for ~15 min. TdT (1:20 dilution in labeling reaction mix) was then added to bind to 3'-OH ends of DNA fragments and catalyze the addition of biotin-labeled and unlabeled deoxynucleotides. Each specimen was covered with Parafilm to prevent evaporation and incubated at 37°C for 1.5 h. Negative controls were incubated with deionized water (1:20 dilution in labeling reaction mix) instead of TdT.

A stop buffer was added for 5 min at room temperature to terminate the labeling reaction. Each slide was then treated with blocking buffer for 10 min. A streptavidin-horseradish peroxidase conjugate (diluted 1:50 in blocking buffer) was added to each slide for 30 min to detect biotinylated nucleotides. The chromagen DAB was used to visualize the labeled 3'-OH end of DNA fragments. H₂O₂ was combined with DAB so that horseradish peroxidase produced a brown precipitant. The slides were then rinsed in deionized water and lightly counterstained with hematoxylin and dehydrated, and coverslips were attached with Permount. Labeling indexes were determined as described in Immunohistochemistry.

**Statistical Analysis**

Differences in serum levels of estradiol and progesterone in each group compared with the control group were analyzed using the Wilcoxon rank-sum test (29). The Wilcoxon rank-sum test was also utilized to compare organ weights and the diameter of the mammary gland duct in the experimental groups with the control group. A two-tailed Fisher’s exact test (30) was employed to compare presence of casein (positive vs. negative expression) between the control group and the experimental groups. In addition, casein expression relative to 28S rRNA and the percentage of cells positive for PCNA and apoptosis in the experimental groups were compared with the control group using the Wilcoxon rank-sum test.

**Results**

This study was designed to determine possible mechanisms that modify mammary carcinogenesis through acute moderate reductions in body weight gain initiated around the time of carcinogen treatment. The average body weight of the food-restricted animals was matched to the average body weight of the 4-HPR-treated animals (Fig. 1). On termination of the study (71 days of age), average food consumption for control, 4-HPR-treated, and food-restricted animals was 17.0, 16.4, and 16.0 g, respectively. The restriction of body weight gains did not affect the survival of the rats or cause any gross signs of toxicity. Estrous cycles were not affected in any group by the changes in body weight.

![Figure 1](https://example.com/figure1.jpg)  
*Figure 1.* Average body weight of rats fed Teklad (4%) diet ad libitum, rats treated with 4-hydroxyphenylretinamide (4-HPR), and rats food restricted to match body weights of those treated with 4-HPR. Food restriction and 4-HPR treatment were initiated at 43 days of age. Methylnitrosourea (MNU, 50 mg/kg body wt) was administered at 50 days of age.
Effect of Reductions in Body Weight Gain on Mammary Gland Development in Female Sprague-Dawley Rats Treated With Methylnitrosourea

Table 1. Effect of Reductions in Body Weight Gain on Serum Levels of Estradiol and Progesterone$^{a,b}$

<table>
<thead>
<tr>
<th>Group</th>
<th>Estradiol, pg/ml</th>
<th>Progesterone, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>43–50 days of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.35 ± 1.07</td>
<td>4.49 ± 0.39</td>
</tr>
<tr>
<td>2</td>
<td>5.87 ± 1.51*</td>
<td>1.79 ± 0.21*</td>
</tr>
<tr>
<td>3</td>
<td>5.08 ± 0.75*</td>
<td>4.44 ± 0.54</td>
</tr>
<tr>
<td>43–57 days of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.75 ± 1.27</td>
<td>7.88 ± 3.13</td>
</tr>
<tr>
<td>2</td>
<td>4.45 ± 0.46</td>
<td>1.50 ± 0.22*</td>
</tr>
<tr>
<td>3</td>
<td>4.91 ± 0.70</td>
<td>4.27 ± 1.00</td>
</tr>
<tr>
<td>43–71 days of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.85 ± 1.24</td>
<td>3.98 ± 0.50</td>
</tr>
<tr>
<td>2</td>
<td>6.70 ± 0.95</td>
<td>1.23 ± 0.12*</td>
</tr>
<tr>
<td>3</td>
<td>5.69 ± 1.02</td>
<td>5.04 ± 0.66</td>
</tr>
</tbody>
</table>

a: Values are means ± SE of 10 rats/group. Group 1 was fed Teklad (4%) diet ad libitum; Group 2 was treated with 4-hydroxyphenylretinamide (4-HPR, 782 mg/kg diet) beginning at 43 days of age; Group 3 was food restricted beginning at 43 days of age to match body weights of animals in Group 2. Methylnitrosourea (50 mg/kg body wt) was administered at 50 days of age.

b: Statistical significance is as follows: *, significantly different from Group 1, $P < 0.05$ (Wilcoxon rank-sum test).

At 50 days of age, after 1 wk of 4-HPR supplementation or dietary restriction, serum levels of estradiol and progesterone were significantly lower ($P = 0.05$ and 0.0001, respectively) in the 4-HPR-treated animals than in the controls (Table 1). Also, at 50 days of age, serum levels of estradiol were significantly ($P = 0.02$) lower in the food-restricted animals than in the controls. At 57 days of age, after 2 wk of 4-HPR supplementation, serum levels of progesterone in 4-HPR-treated animals ($P = 0.005$) were reduced compared with controls. Similarly, at 71 days of age, after 4 wk of 4-HPR supplementation, serum levels of progesterone were significantly lower ($P ≤ 0.0001$) in 4-HPR-treated animals than in controls. No significant differences in the levels of serum estradiol or progesterone at 50 or 71 days of age were observed between control and food-restricted animals.

Liver, uterus, and adrenal weights were measured at 50, 57, and 71 days of age. No significant differences were observed in any of the organ weight measurements in 4-HPR-treated and food-restricted animals compared with controls at 50 days of age. At 57 days of age, the uterus weight was lower for 4-HPR-treated animals than for controls ($P = 0.03$). The adrenal weights at 57 days of age were higher for 4-HPR-treated and food-restricted animals than for controls ($P = 0.001$ and 0.03, respectively). At 71 days of age, the average liver weight was higher for 4-HPR-treated animals than for controls ($P = 0.006$).

For controls at 50 and 71 days of age, there were ~70–75% terminal end buds and 25–30% alveolar buds in each mammary whole mount; at 71 days of age, there were ~60–75% alveolar buds and 25–40% terminal end buds. Mammary glands in 4-HPR-treated animals showed a striking decrease in size of alveolar buds and terminal end buds from 50 to 71 days of age compared with controls. Also, in 4-HPR-treated animals, it was apparent that several terminal end buds involuted to terminal ducts. Mammary glands from food-restricted and control animals were similar from 50 to 71 days of age, showing similar proportions of terminal end buds and alveolar buds for each time period as described above. Although the proportion of structures within mammary glands of food-restricted animals was similar to that of controls, these glands appeared to be smaller in size than in controls. The average diameter of the mammary gland duct and the number and diameter of terminal end buds, alveolar buds, and terminal ducts per random field were determined for each group at 71 days of age (Table 2). A significant decrease in the diameter of the mammary gland duct was observed in 4-HPR-treated ($P = 0.03$) and food-restricted animals ($P = 0.03$) compared with controls. Figure 2 shows significant suppression of mammary gland structures and differentiation in 4-HPR-treated animals and slight suppression of mammary gland structures in food-restricted animals compared with ad libitum-fed animals.

Northern blot analysis of casein expression was used to quantitate differentiation in ad libitum-fed, 4-HPR-treated, and food-restricted animals at 50, 57, and 71 days of age. RNA was isolated from five inguinal/abdominal mammary gland samples from each group for each time period. Each of

Table 2. Effect of Moderate Reductions in Body Weight Gain on Mammary Gland Development in Female Sprague-Dawley Rats Treated With Methylnitrosourea$^{a,b}$

<table>
<thead>
<tr>
<th>Group</th>
<th>Terminal End Buds</th>
<th>Alveolar Ducts</th>
<th>Terminal Ducts</th>
<th>Diam of Mammary Gland Ducts,$d$ mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage</td>
<td>Diam,$c$ mm</td>
<td>Percentage</td>
<td>Diam,$c$ mm</td>
</tr>
<tr>
<td>1</td>
<td>26.0</td>
<td>7.3</td>
<td>65.7</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>22.5</td>
<td>7.8</td>
<td>22.9</td>
<td>14.2</td>
</tr>
<tr>
<td>3</td>
<td>38.8</td>
<td>6.9</td>
<td>49.7</td>
<td>13.2</td>
</tr>
</tbody>
</table>

a: Methylnitrosourea (50 mg/kg body wt) was administered at 50 days of age.

b: Statistical significance is as follows: *, significantly different from Group 1, $P < 0.05$ (Wilcoxon rank-sum test).

c: Average diameter in a random field of 5 rats/group.

d: Values are means ± SE of 50 measurements (10 measurements/animal).
the RNA samples was subjected to Northern blot analysis and labeled with a 32P-labeled casein probe and a 32P-labeled 28S rRNA probe. Autoradiographs showing levels of casein and 28S rRNA (loading controls) expression for ad libitum-fed, 4-HPR-treated, and food-restricted animals at 50, 57, and 71 days of age are shown in Fig. 3.

An increase in the mean level of casein expression relative to 28S rRNA was observed in food-restricted compared with ad libitum-fed animals at 50 days of age (Fig. 3) using a Wilcoxon rank-sum test. However, this increase was skewed because of a strong level of casein expression in one of the samples. Positive vs. negative expression of casein was also compared across all groups for each time period using a Fisher’s exact test (2-tail). Casein expression (positive vs. negative) in food-restricted animals was not significantly different from that in ad libitum-fed animals at 57 and 71 days of age ($P = 0.05$ and 0.05, respectively).

The percentage of cells positive for apoptosis and PCNA was also determined in 10 animals in each group for each time period. Table 3 includes the labeling indexes for the percentage of cells positive for apoptosis in ad libitum-fed, 4-HPR-treated, and food-restricted animals at 50, 57, and 71 days of age. Our results indicated no significant differences when 4-HPR-treated and food-restricted animals were compared with ad libitum-fed animals. Table 4 presents the labeling indexes for cells expressing the PCNA antigen in ad libitum-fed, 4-HPR-treated, and food-restricted animals at each time period. In 4-HPR-treated animals, there appeared to be a trend toward a decrease in cells expressing the PCNA antigen compared with ad libitum-fed animals at 57 and 71 days of age.

**Discussion**

This study was designed to better understand the inhibitory effects of moderate dietary restriction initiated around the time of mammary cancer induction. For each time period, the levels of estradiol were decreased in the 4-HPR-treated and food-restricted groups compared with controls. At 50 days of age, 1 wk after initiation of 4-HPR supplementation or dietary restriction, the serum levels of estradiol were significantly lower than in ad libitum-fed animals. Estradiol levels were lower than in ad libitum-fed animals during a short period of time, and this finding correlates with previously cited reports that suggest estrogen’s role as a risk factor for mammary carcinogenesis (31–33) and supports the observation of Sylvester et al. (32) that calorie restriction decreases estrogen levels.

Engelman et al. (34) reported a reduction in cellular proliferation after analyzing mammary gland whole mounts from mice restricted by 40% of calories from controls. Although the analysis of mammary gland whole mounts indicated that at 71 days of age mammary glands of the dietary-restricted animals were similar in the proportion of structures compared with the control group (Table 2), significant decreases ($P = 0.03$) in the diameter of the mammary gland ducts between food-restricted and ad libitum-fed animals were observed. The mammary gland in the dietary-restricted group showed a slight decrease in the size of mammary gland structures, which may indicate a slight decrease in proliferation. Although previous studies (20,37,38) have shown that calorie restriction (restrictions of 10–40%) may enhance apoptosis, our findings did not. Other studies have also shown in other tissues that the percentage of cells positive for PCNA was decreased in calorie-restricted (restrictions of 35%, 40%, and 64%) animals (38–40). Our findings for PCNA showed a significant decrease in the percentage of cells positive for PCNA in the dietary-restricted group only at 57 days of age. Thus it is possible that, to induce biologically significant effects on proliferation and apoptosis, di-
etary restrictions must be >15%. Our results observed with β-casein expression, a biomarker of differentiation, suggest that the inhibitory action on mammary cancer of reduced body weight gain might not be entirely related to changes in differentiation of the mammary gland. Although it was not the focus of this study, the results obtained from the 4-HPR group have proved interesting. Few data are available correlating levels of progesterone after 4-HPR administration. The role of progesterone in reduced mammary carcinogenesis induced by 4-HPR has not been elucidated. Our results on the effect of 4-HPR on mammary gland development are similar to those reported by Moon et al. (35). The mammary glands of 4-HPR-treated animals showed a remarkable decrease in the diameter of mammary gland ducts and an increase in differentiation (increase in terminal ducts) compared with controls. There was also a marked decrease in the percentage of cells positive for PCNA in the 4-HPR-treated group compared with controls at 57 and 71 days of age.

β-Casein was only slightly expressed in the 4-HPR-treated group compared with the controls, which contrasts with our mammary gland whole mount findings. Our seemingly contrasting data can be explained by the changes in mammary development. Mammary gland differentiation is typically defined as a loss of terminal end buds and the occurrence of alveolar buds and alveoli (during lactation). However, a mammary gland that involutes from terminal

Figure 3. Autoradiographs showing 32P-labeled β-casein mRNA and 28S rRNA expression in rat mammary gland at 50, 57, and 71 days of age. Lanes 1–5, Teklad (4%) diet ad libitum; Lanes 6–10, 4-HPR; Lanes 11–15, dietary restriction to match body weights of rats treated with 4-HPR. A, B, and C: β-casein mRNA expression in rat mammary gland at 50, 57, and 71 days of age, respectively. D, E, and F: 28S rRNA expression in rat mammary gland at 50, 57, and 71 days of age, respectively.
end buds to terminal ducts is also considered to be more differentiated (i.e., simply because terminal end buds are not present). β-Casein is often used as a biomarker of differentiation, because β-casein is expressed at low levels in virgin rats but amplified during early pregnancy (36). Thus β-casein is a good biomarker for this type of differentiation in which lactation is the end point. We have shown that dehydroepiandrosterone, a compound that differentiates the mammary gland similar to that of pregnancy and lactation, caused high levels of β-casein (unpublished data) to be expressed. The 4-HPR-treated animals clearly showed a loss of terminal end buds and an increase in terminal ducts. Thus mammary development was more differentiated in the 4-HPR-treated animals than in the controls. However, β-casein (a biomarker for differentiation) was not expressed significantly in the 4-HPR-treated rats. Thus a protein that is characteristic of mammary gland ducts, rather than alveoli, would be a more appropriate biomarker of differentiation for 4-HPR and would explain our observations. Although β-casein is considered to be an indicator of mammary differentiation, the type of differentiation that occurs in the mammary gland must be considered carefully.

The effects we observed for each parameter were measured around the time of carcinogen administration and, thus, were from normal or recently initiated cells. Biologically significant effects of 4-HPR treatment and dietary restriction may be more pronounced in fully transformed cells. Our previous studies (1,2) demonstrated that moderate reductions in body weight gain, as observed after administration of some chemopreventive agents, must be considered in the design of chemoprevention studies. The experimental evidence is lacking to support a mechanism responsible for mediating decreased tumorigenesis as observed with a reduction in body weight gain from chemopreventive agents or dietary restriction. In the present study, dietary restriction and chemopreventive treatment induced different effects on morphological, endocrine, and biochemical indexes. Thus it is possible that the decreased mammary tumorigenesis induced by body weight gain reduction from dietary restriction and chemopreventive agents might be mediated through different mechanisms.

### Acknowledgments and Notes

The authors thank the technicians for their excellent work, Ina Baswell for help with histology services, and Stephanie Pendergrass for assistance with the mammary gland whole mount analyses. This investigation was supported by National Cancer Institute Contract Numbers N01-CN-65025-MAO and N01-CN-75101-MAO. Address correspondence to C. Rodríguez-Burford, BDB 11, 1530 3rd Ave. South, Birmingham, AL 35294-0012. Phone: (205) 975-6545. FAX: (205) 975-6522. E-mail: cburford@uab.edu.

Submitted 8 November 2001; accepted in final form 7 March 2002.

### References


### Table 3. Percentage of Cells Positive for Apoptosis

<table>
<thead>
<tr>
<th>Group</th>
<th>50 days of age</th>
<th>57 days of age</th>
<th>71 days of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.16 ± 0.58</td>
<td>6.57 ± 0.97</td>
<td>8.31 ± 2.47</td>
</tr>
<tr>
<td>2</td>
<td>4.54 ± 0.66</td>
<td>9.73 ± 2.41</td>
<td>6.24 ± 0.89</td>
</tr>
<tr>
<td>3</td>
<td>7.90 ± 2.06</td>
<td>9.99 ± 2.37</td>
<td>5.07 ± 1.17</td>
</tr>
</tbody>
</table>

a: Values are means ± SE of 10 rats/group.

### Table 4. Percentage of Cells Expressing PCNA

<table>
<thead>
<tr>
<th>Group</th>
<th>50 days of age</th>
<th>57 days of age</th>
<th>71 days of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.15 ± 2.28</td>
<td>15.9 ± 3.52</td>
<td>7.10 ± 1.94</td>
</tr>
<tr>
<td>2</td>
<td>10.9 ± 2.58</td>
<td>8.08 ± 2.65</td>
<td>2.74 ± 1.14</td>
</tr>
<tr>
<td>3</td>
<td>12.8 ± 2.86</td>
<td>12.2 ± 3.08</td>
<td>8.78 ± 3.32</td>
</tr>
</tbody>
</table>

a: Values are means ± SE of 10 rats/group.


