

Effect of Energy Restriction on the Expression of Cyclin D1 and p27 During Premalignant and Malignant Stages of Chemically Induced Mammary Carcinogenesis

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The restriction of energy intake has a profound inhibitory effect on carcinogenesis, yet the mechanism or mechanisms that account for this effect are unknown. In this experiment, the hypothesis tested was that energy restriction upregulates the expression of p27/kip1, a gene product associated with cell-cycle growth arrest, while downregulating cyclin D1, a protein that combines with cyclin-dependent kinases to promote phosphorylation of retinoblastoma protein and the progression of cells through the cell cycle. We studied levels of these proteins in uninvolved mammary epithelial cells and in mammary intraductal proliferations, ductal carcinomas *in situ*, and adenocarcinomas induced in response to administration of 1-methyl-1-nitrosourea in animals fed either *ad libitum* or 90%, 80%, or 60% of *ad libitum* intake. Protein levels were evaluated immunohistochemically by using computer-assisted image analysis to quantify differences in protein expression among treatment groups. The expression of p27 increased and the expression of cyclin D1 decreased dose-dependently in response to energy restriction. The effect was greater on p27 than on cyclin D1. The hypothesis proposed is that energy restriction inhibits carcinogenesis by arresting cell-cycle progression by regulating p27/kip1. *Mol. Carcinog.* 24:241–245, 1999. © 1999 Wiley-Liss, Inc.

Key words: energy restriction; mammary carcinogenesis; cyclin D1; p27/kip1; cell-cycle progression

INTRODUCTION

Restricting the energy to an organism, without significantly affecting the availability of other nutrients, has been shown in numerous model systems to profoundly inhibit the development of experimentally induced cancer [1]. This effect is potentially important because it is accompanied by other benefits, including prolonged survival, enhanced immunological activity, and reduced incidence of other chronic diseases [2–4]. Despite the voluminous literature on this topic, little is known about what accounts for the cancer-protective activity of energy restriction.

Our laboratory recently published evidence that energy restriction increases the activity of the adrenal cortical steroid pathway, as was reflected by increased excretion of cortical steroid reactive substances in urine, and that irrespective of the level of energy restriction to which the animals were exposed (range, 0–40% restriction), the excretion of urinary cortical steroids accounted for the variance in tumor multiplicity among the animals [5]. These results suggest a cause-and-effect relationship. The finding that urinary cortical steroid excretion increased with increasing energy restriction is consistent with the predicted metabolic consequences to an animal in response to a limitation of available energy, namely that the glucocorticoid–insulin (and related growth factors) axis will be tipped in the direction of upregulated cortical steroid activity, with

a concomitant downregulation of insulin and its related family of peptides, their receptors, or both [6]. We have juxtaposed these observations with a line of evidence that cell proliferation is decreased and cell death caused by apoptosis is increased in energy-restricted animals. Although it is tempting to speculate that the effects of energy restriction on cell proliferation and cell death account for the cancer-inhibitory activity of energy restriction, analysis of this idea suggests that such alterations probably reflect the mechanism by which energy restriction inhibits carcinogenesis but do not in themselves account for its protective activity.

Currently, a great deal of research is focused on defining the mechanisms by which cells enter, transit, and exit the cell cycle and on identifying the specific steps in these processes that are deregulated during carcinogenesis [7]. In view of the evidence that energy restriction appears to affect parameters related to cell-cycle progression, we used paraffin-embedded tissues from our previous experiment [5] to ask, Are there specific genes involved in cell-cycle

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Abbreviations: RF, restricted-fed; DAB, diaminobenzidine; cdk, cyclin-dependent kinase.

transit that are regulated by imposition of energy restriction and its effect on cortical steroid-insulin metabolism? Data from this study indicated that the expression of two gene products involved in cell-cycle progression, cyclin D1 and p27/kip1, was affected by energy restriction in a dose-dependent manner.

MATERIALS AND METHODS

Carcinogen Administration and Diets

Female Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) at 20 d of age. At 21 d of age, the animals were given 50 mg of 1-methyl-1-nitrosourea/kg of body weight intraperitoneally as previously described [8], and they were randomly divided into the following groups (25 rats/group): ad libitum-fed (control), restricted-fed (RF) to 90% of control (90% RF), RF to 80% of control (80% RF), and RF to 60% of control (60% RF). A modified AIN-93G diet and feeding protocol were used as previously described [5]. The diets fed to energy-restricted animals were formulated to ensure an equivalent intake of all nutrients while limiting total dietary calories by reducing carbohydrate. All rats were fed two meals per day (at 6:00–9:00 AM and 2:00–5:00 PM), 7 d/wk to avoid possible confounding due to intergroup variation of meal timing, meal number, and duration of fasting. The amount of food fed the RF rats was calculated every 2 d based on the amount consumed by the control group during the preceding 2 d. The rats were housed individually in stainless-steel metabolic cages with wire-mesh bottoms. The cages were equipped with adjustable-width external tunnel feeders that permitted accurate quantification of food intake. The rooms were maintained at $22 \pm 1^\circ\text{C}$ with 50% relative humidity and a 12-h light/12-h dark cycle. All rats were weighed three times per week. The animal facility in which the rats were housed is accredited by the American Association for the Accreditation of Laboratory Animal Care. The experiment was reviewed and approved by the AMC Cancer Research Center Institutional Animal Care and Use Committee.

Necropsy

All rats were killed 5 wk after carcinogen administration by using a stratification sequence so that the time from last meal and the time of day at which death occurred were equally represented in all groups. The abdominal-inguinal mammary glands from all animals were carefully excised and prepared as whole mounts as previously described [8]. Lesions identified in the whole mounts were processed for histopathological classification [9] and immunohistochemical analysis.

Staining and Quantification of Cyclin D1 and p27

Uninvolved mammary gland duct and premalignant and malignant mammary gland lesions were

evaluated. It should be noted that the premalignant and malignant mammary gland lesions occurring in energy-restricted rats represent lesions resistant to inhibition by energy restriction, and they may not fully reflect the effects of energy restriction on carcinogen-treated cells that failed to progress. The lesions were stained for immunohistochemical analysis of cyclin D1 or p27. Anti-cyclin D1 antiserum (diluted 1:40) and anti-p27 antiserum (diluted 1:70) (NeoMarkers, Union City, CA) were used to detect cyclin D1-labeled and p27-labeled nuclei. Labeled and unlabeled cells were imaged and counted at 400 \times with a computer-assisted image analyzer (CAS-200) by using the Quantitative Nuclear Antigen Program version 3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA) [10]. Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with hematoxylin, with or without diaminobenzidine (DAB) staining, and the other at 500 nm, which measures only nuclei stained with DAB, allowed excellent spectral discrimination between the brown (DAB chromogen) and blue (hematoxylin chromogen). Both nuclear and antibody thresholds were set with a negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between the nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide. Standardization was established by using the same control tumor tissue in each assay, which was treated in the same manner as the sample tissue.

The Quantitative Nuclear Antigen software application was used to measure the percentage of cell nuclei in the tissue sections that contained cyclin D1- or p27-labeled antigen by counting 20 fields per slide (approximately 2000 cells). The optical density data obtained were used to compute the percentage of cells that expressed a particular protein and the estimated amount of protein per positive cell. Ten independent specimens were evaluated for each type of lesion in each dietary group.

Statistical Analyses

Data are reported as means \pm standard errors of the means. Because immunohistochemical staining for a protein is not stoichiometric relative to the amount of protein present, differences in staining were analyzed by using a Kruskal-Wallis rank test [11].

RESULTS

The tissue sections evaluated in this study were obtained from the paraffin blocks prepared for histological analyses of specimens used to determine whether energy restriction results in a dose-dependent reduction in the incidence and multiplicity of mammary ductal carcinomas in situ and adenocarcinomas [5].

Figure 1A and B shows a reduction in cyclin D1-

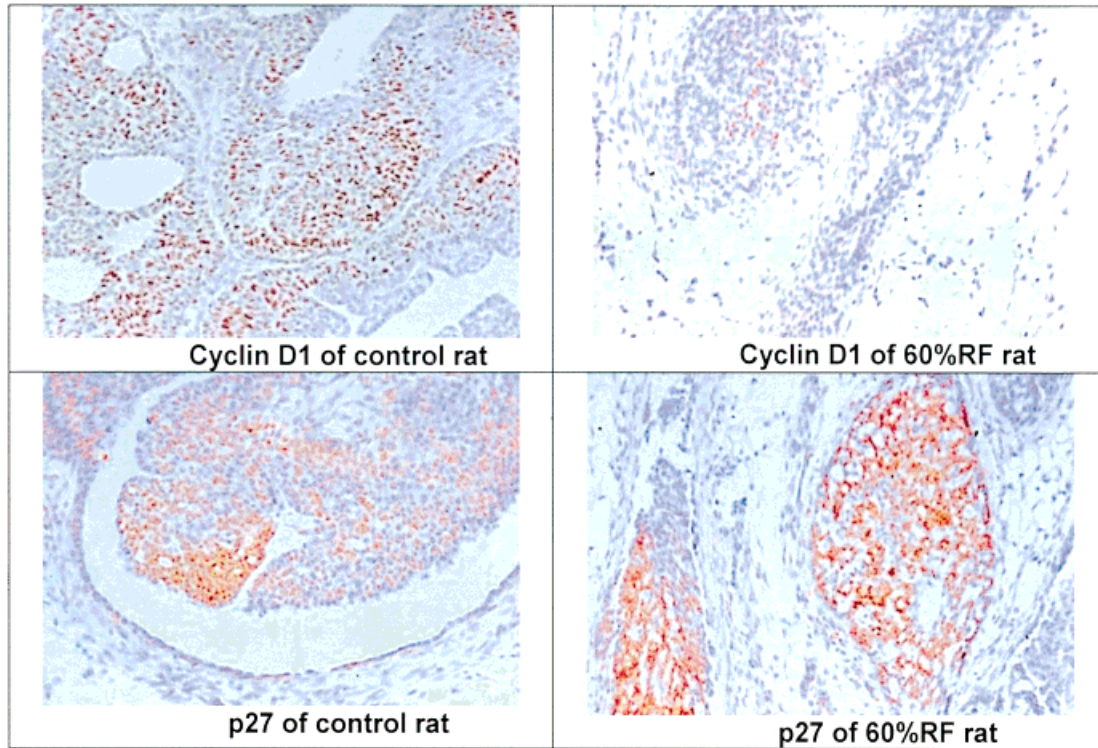


Figure 1. Immunohistochemical staining for cyclin D1 and p27 in mammary adenocarcinomas of ad libitum-fed control rats (A and C) and 60% RF rats (B and D).

specific immunostaining in mammary adenocarcinomas excised from an energy-restricted rat (Figure 1B) in comparison with an ad libitum-fed rat (Figure 1A). The degree to which energy restriction reduced the percentage of mammary epithelial cells staining positive for cyclin D1 in ducts, intraductal proliferations, ductal carcinomas in situ, and adenocarcinomas is shown in Table 1. In ad libitum-fed (control) rats, a dramatic increase in the number of cells expressing cyclin D1 was observed in adenocarcinomas relative to other types of lesions. The only statistically significant effect of energy restriction was on the overexpression of cyclin D1 observed in carcinomas. The two highest levels of

energy restriction resulted in a proportion of cyclin D1-positive cells in adenocarcinomas similar to that observed in intraductal proliferations and ductal carcinomas in situ.

Figure 1C and D shows an increase in p27-specific immunostaining in mammary adenocarcinomas excised from an energy-restricted rat (Figure 1D) in comparison with an ad libitum-fed rat (Figure 1C). The degree to which energy restriction increased the percentage of mammary epithelial cells staining positive for p27/kip1 in ducts, intraductal proliferations, ductal carcinomas in situ, and adenocarcinomas is shown in Table 2. Less than 1% of mammary epithelial cells in carcinomas was observed to express p27.

Table 1. Effect of Energy Restriction on Percentage of Cells Staining Positive for Cyclin D1*

Lesion [†]	Control [‡]	90% RF	80% RF	60% RF	P value
Duct	2.41 ± 0.78	1.42 ± 0.16	1.08 ± 0.24	1.00 ± 0.16	0.27
IDP	13.58 ± 2.57	11.72 ± 0.82	10.61 ± 1.98	10.22 ± 3.76	0.72
DCIS	16.59 ± 3.47	13.15 ± 1.57	10.40 ± 0.60 [§]	10.68 ± 2.90 [§]	0.68
AC	40.58 ± 4.02	34.70 ± 4.57	23.97 ± 2.93 [§]	23.12 ± 2.56 [§]	0.04

*Approximately 2000 cells were evaluated in each lesion. Each value is a mean ± standard error of the mean for 10 independent lesions. The data were analyzed by the Kruskal-Wallis rank test. The P values are for a trend analysis across RF levels.

[†]Duct, ductal mammary epithelial cells in which no neoplastic changes were observed; IDP, intraductal proliferations; DCIS, ductal carcinoma in situ; AC, adenocarcinomas.

[‡]Control animals were fed ad libitum.

[§]P < 0.05, compared with the control group.

^{||}P < 0.05, compared with the 90% RF group.

Table 2. Effect of Energy Restriction on Percentage of Cells Staining Positive for p27*

Lesion [†]	Control [‡]	90% RF	80% RF	60% RF	P value
Duct	4.04 ± 0.86	12.27 ± 1.32 [§]	23.61 ± 4.06	24.31 ± 3.37	0.001
IDP	3.97 ± 2.20	11.30 ± 3.06 [§]	17.92 ± 4.06 [§]	24.57 ± 5.95 [§]	0.003
DCIS	4.91 ± 1.96	16.04 ± 3.82 [§]	23.70 ± 9.09 [§]	33.59 ± 6.94 ^{§ ¶}	0.012
AC	0.65 ± 0.42	4.55 ± 0.63 [§]	6.31 ± 1.51 [§]	8.42 ± 3.48 [§]	0.001

*Approximately 2000 cells were evaluated in each lesion. Each value is a mean ± standard error of the mean for 10 independent lesions. The data were analyzed by the Kruskal-Wallis rank test. The P values are for a trend analysis across RF levels.

[†]Duct, ductal mammary epithelial cells in which no neoplastic changes were observed; IDP, intraductal proliferations; DCIS, ductal carcinoma in situ; AC, adenocarcinomas.

[‡]Control animals were fed ad libitum.

[§]P < 0.05, compared with the control group.

^{||}P < 0.05, compared with the 90% RF group.

[¶]P < 0.05, compared with the 80% RF group.

This percentage was much lower than that observed in mammary epithelial cells from ducts (6.2-fold difference), cells in intraductal proliferations (6.1-fold difference), or ductal carcinomas in situ (7.6-fold difference). Energy restriction resulted in dose-dependent increases in the proportions of cells expressing p27. The magnitude of increased expression ranged from approximately 3- to 20-fold.

DISCUSSION

The hypothesis evaluated in this study was that limiting energy induces specific effects on the cell cycle that limit overall growth as well as growth related to the development of malignancy. Based on reports that cortical steroids can regulate the transcription and translation of cyclin D1 and p27, respectively [12,13], we investigated the effects of energy restriction on the levels of these two proteins in cells involved in mammary carcinogenesis. The results showed that the number of cells expressing cyclin D1 protein was decreased and the number expressing p27 protein was increased dose-dependently by energy restriction. Clearly, as immunohistochemical analyses showed, the magnitude of the effect of energy restriction on p27 protein expression greatly exceeded the effect on cyclin D1. However, as p27 has been reported to complex with cyclin D–cyclin-dependent kinase (cdk) to inhibit cell-cycle progression, immunoprecipitation studies are needed to further characterize the response we observed. To our knowledge, this is the first report of an effect of energy restriction on the cellular level of either cyclin D1 or p27 protein.

That energy restriction has a dominant effect on the cellular levels of p27 protein may indicate a cause-and-effect relationship. Among the intriguing aspects of the finding is that loss of p27 protein expression in p27 knockout mice results in organomegaly, a result that would be predicted if p27 played an important role in maintenance of tissue size [14,15]. As reported elsewhere, our laboratory and others have hypothesized that carcinogenesis represents a fundamental loss of regulatory control of tissue-size homeostasis [16,17]. Thus, the profound protective

effect of energy restriction on carcinogenesis is consistent with our hypothesis that energy restriction exerts its effect via the upregulation of a protein that is involved in regulating size homeostasis. As noted above, p27 is an inhibitor of the cyclin D1–cdk4 and cyclin E–cdk2 complexes [18,19], which, as mounting evidence indicates, contribute to dysregulation in mammary carcinogenesis [20]. Thus, energy restriction may work by delaying the entry of carcinogen-initiated cells as well as their nontransformed counterparts into the cell cycle. The simplicity of this candidate mechanism is appealing, and it implies that the induction of p27 could be an important molecular target of cancer prevention strategies.

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