

Effect of corticosterone administration on mammary gland development and p27 expression and their relationship to the effects of energy restriction on mammary carcinogenesis

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The inhibitory activity against mammary carcinogenesis mediated by energy restriction is accompanied by a reduction in the degree of mammary ductal branching, and an increase in adrenal cortical activity. Levels of p27/kip1 protein, a gene product associated with cell cycle growth arrest, have also been shown to be elevated in mammary epithelium and in mammary lesions of energy-restricted animals. Based on these data we have proposed that increased secretion of adrenal cortical steroids accounts, in part, for the effects of energy restriction. In this experiment the hypothesis tested was that corticosterone administration would mimic the effects of energy restriction, both on mammary gland development and on levels of p27 protein in mammary ductal epithelium. To test this hypothesis corticosterone was fed to female rats for 4 weeks. Dietary corticosterone increased serum and urinary corticosterone levels in a dose-dependent manner ($P < 0.01$). The effects of corticosterone treatment on mammary gland development were analyzed digitally; p27 protein was detected immunohistochemically. The ductal extension and branching of the mammary gland were reduced in a dose-dependent manner by corticosterone treatment ($P < 0.05$); however, the magnitude of the effect was greater on ductal branching. Overall, increasing dietary corticosterone reduced the total volume of mammary epithelium in a dose-dependent manner, an effect that remained even after adjustments for differences among animals in body mass. Consistent with this effect, the amount of p27 protein present in ductal mammary epithelial cells increased dose-dependently in response to increasing corticosterone administration ($P < 0.01$). The hypothesis is proposed that dietary administration of corticosterone may imitate the effects of energy restriction on mammary carcinogenesis by regulation of mammary tissue size homeostasis via p27/kip1 mediated arrest of cell cycle progression.

Introduction

We have recently reported that feeding a nutritionally adequate diet that was restricted in energy enhanced adrenal cortical activity as reflected by increased excretion of cortical steroid reactive substances in urine. The extent of increased cortical steroid activity was directly related to the inhibition of mammary carcinogenesis by energy restriction (1). A substantial

Abbreviations: cdk, cyclin-dependent kinase; Corti-50, 50 mg corticosterone/kg diet; Corti-100, 100 mg corticosterone/kg diet; Corti-200, 200 mg corticosterone/kg diet; Corti-400, 400 mg corticosterone/kg diet; DAB, diaminobenzidine.

body of literature characterizes the role of the adrenal gland in concert with other endocrine organs in mediating the host response to energy restriction (2–4). Thus the literature supports a direct relationship between adrenal cortical steroids and inhibition of mammary carcinogenesis (1,5). Adrenal cortical steroids are involved in mammary gland growth and development (6–8). These steroids control a variety of anabolic and catabolic processes and play a fundamental role in the regulation of proliferation, death and differentiation (9). Adrenalectomy has been reported to accelerate mammary carcinogenesis whereas injection of pharmacological levels of cortical steroids inhibits the process (5,10). There is increasing recognition that cortical steroids may exert their effects, at least in part, by directly regulating either entry into or progression of cells through the cell cycle (11). Progression through the cell cycle is mediated by the activation of the cyclin-dependent kinases (cdks), which are regulated by complex formation with a cyclin partner and by phosphorylation at specific residues (12). Two families of proteins act as cdk inhibitors (12,13). They are the p16 family (p16/Ink4, p15, p18 and p19) and the p21 family (p21, p27 and p57). Given reports that adrenal cortical steroids may regulate the activity of p27 (14), the work reported in this paper focused on this gene.

Based on the evidence that energy restriction appears to have effects on mammary gland development and cell cycle progression, and that these effects may be mediated by adrenal cortical steroids, the objective of the experiment reported in this study was to determine whether mammary gland development and levels of p27 protein could be regulated by increasing serum cortical steroid concentrations. In this paper, an experimental approach for the investigation of the role of adrenal steroids in mediating the protective effects of energy restriction is defined, and data are reported that indicate that mammary gland development was arrested and the amount of p27/kip1 protein was increased by corticosterone treatment.

Materials and methods

Corticosterone administration and diet

Forty female Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY) at 20 days of age. One week later, animals were randomly divided into five groups (eight rats/group) and fed one of the following diets for 4 weeks: modified AIN-93G (1) (control), or the AIN-93G supplemented with corticosterone at 50, 100, 200 and 400 mg corticosterone/kg diet. Rats were housed individually in stainless steel cages with wire mesh bottoms. The animal room was maintained at $22 \pm 1^\circ\text{C}$ with 50% relative humidity and a 12 h light–dark cycle. All rats were weighed twice per week. The animal facility in which the rats were housed is AAALAC accredited. This experiment was reviewed and approved by the AMC Cancer Research Center Institutional Animal Care and Use Committee.

Assessment of cortical steroid status

Twenty-four hour collections of urine were obtained before and after the 4 week feeding study. Blood was collected in microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) from the tail between 9:00–11:00 a.m. on the day the study was terminated. During blood collection, the animals were kept under quiet and comfortable conditions in a heating pad without anesthesia to diminish extra stimulation which could influence the secretion of cortical steroids. Two persons assisted with blood collection, which took ~1 min/rat.

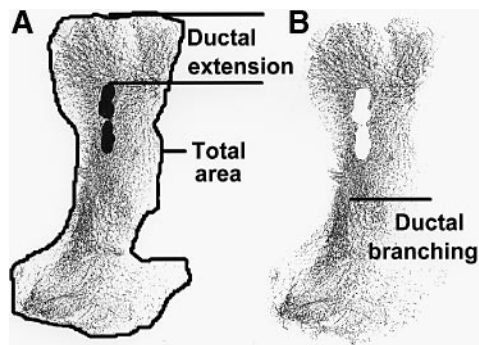


Fig. 1. Illustration of mammary gland growth measurements. (A) Ductal extension is the distance between the superior-most lymph node and the mammary branch border; total area is the area of fat pad into which mammary terminal end ducts extended, from which the area occupied by the lymph nodes was subtracted. (B) The area occupied by mammary gland epithelium.

Urine and serum separated by centrifugation were assayed for immunoreactive corticosteroids using an antisera specific for corticosterone via a direct radioimmunoassay procedure (ICN Biomedicals, Costa Mesa, CA) as described previously (1). The urinary corticosteroids were expressed as $\mu\text{g}/\text{kg}$ body wt/day and the serum corticosteroids were expressed as ng/ml.

Necropsy

All rats were killed at the end of study that was terminated after feeding the corticosterone-supplemented diets for 4 weeks. At necropsy, adrenal glands were removed and immediately weighed. The abdominal–inguinal mammary glands from all animals were carefully excised and prepared as whole mounts as previously described (15). The whole mounts were processed for image analyses and immunohistochemical staining as described below.

Processing of mammary gland whole mounts for digital image analysis

Digital images of whole mounts of the abdominal–inguinal mammary gland were obtained using a digital camera (Kodak DCS420, Kodak Digital Science, Rochester, NY) with the light source passing through the sample. The images were then downloaded into Adobe Photoshop using a Kodak DCS TWAIN driver. The digitized colored images were transformed into black and white images and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Measurements of ductal extension of the mammary gland into the fat pad, mammary gland size and amount of mammary gland epithelium were performed on the digitized images of entire abdominal–inguinal mammary gland chains. Measurements were calibrated using a ruler photographed with the whole mount image. The ductal extension was expressed in mm, and both total area of the mammary gland and area occupied by mammary gland epithelium were expressed in cm^2 . The results reported are the original size of the mammary gland whole mount.

Measurement of mammary gland growth

As reported before and shown in Figure 1A, the length of mammary gland between the upper-most lymph node and mammary branch border was quantified as a measure of ductal extension. Images were then further processed to remove the lymph nodes from the mammary gland (Figure 1B). These processed images were then evaluated for total area of the mammary gland fat pad into which mammary terminal end ducts had extended. This area was defined by drawing a line around the 360° perimeter of a mammary gland chain established by the point-to-point connection of a line from the outer most extending end bud to the other. The area within this perimeter was then determined by subtracting the area occupied by the lymph nodes.

Assessment of the amount of mammary gland epithelium

The amount of mammary gland epithelium was estimated using two methods. The first approach estimated the area occupied by mammary gland epithelium, which represented a two-dimensional assessment. The second approach measured the mammary gland epithelial density, which estimated the amount of overlapping mammary epithelium based on summed differences in pixel density.

Staining and quantification of p27 protein

Mammary gland ductal epithelium was stained for the immunohistochemical analysis of p27. Antibody of anti-p27 (NeoMarkers, 1:70) was used to detect p27 labeled nuclei. Labeled and unlabeled cells were imaged and counted at $\times 400$ with a computer-assisted image analyzer (CAS-200), using a Quantitative Nuclear Antigen Program Version 3.0 (Becton–Dickinson/Cellular Imaging

Systems, San Jose, CA) as described previously (16). p27 was determined by counting 20 fields/slide (~ 2000 cells/gland).

Statistical analyses

Data were evaluated for their conformity to assumptions of distributional normality: if required, data were log transformed. Normally distributed data were then subjected to analysis of variance and *post hoc* comparisons were made using Tukey's multiple-range test (17). When effects of body weight on mammary gland morphometrics were evaluated, data were subjected to analysis of covariance with body weight as the covariate (17). Data were also subjected to regression analyses for test of trend (17). Immunohistochemical staining is not a stoichiometric process, thus the resulting data were not truly continuous. Because of this, the Kruskal–Wallis rank test was used to evaluate the p27 staining data (18). Systat 7.0 statistical analysis program (Evanston, IL) was used to perform all statistical analyses.

Results

Cortical steroid levels in urine and serum, and adrenal gland weight

The effects of dietary corticosterone treatment on cortical steroids in urine and serum are shown in Table I. Urine was obtained prior to initiating the feeding of the experimental diets and at the end of the experiment. No differences in urinary corticosteroid excretion were found among groups before dietary corticosterone administration. The levels of cortical steroids were increased in both urine and serum with increasing corticosterone in the diet as determined by regression analysis ($P < 0.001$). As anticipated, serum levels of cortical steroids and those measured in the urine were highly correlated ($r = 0.51$, $P < 0.01$). The weight of adrenal glands was significantly decreased with increasing dietary corticosterone ($P < 0.01$) as determined by regression analysis.

Animal growth and mammary gland development

As shown in Table II, all animals in the corticosterone-treated groups gained weight throughout the experiment; however, the rate of gain was somewhat slower with increasing level of cortical steroid (regression analysis $P = 0.003$ for trend of decreased rate of growth).

Figure 2 shows whole mount preparations that were representative of the effects of dietary corticosterone on the development of the abdominal–inguinal mammary gland chain. In order to quantify and evaluate the differences in the development of the mammary gland reflected in Figure 2, the abdominal–inguinal mammary gland chains of all animals in this study were digitized and the digital images were analyzed as described in the Materials and methods section (also refer to Figure 1). The results of these analyses are shown in Table II. Ductal extension of the mammary gland into the fat pad, the area of the fat pad into which mammary gland had extended, the total area occupied by mammary epithelium, and the estimated volume of mammary epithelium within the fat pad were reduced with increasing levels of dietary corticosterone and all reductions were statistically significant ($P < 0.05$). Ductal extension measured either in one direction or as the area of the fat pad into which the mammary gland extended provided an assessment of linear growth of mammary ducts. Although corticosterone reduced ductal growth the effects were, in general, small and were proportional to the effects of corticosterone on body weight gain as determined by regression analyses. The effect of cortical steroids was more pronounced on the lateral branching of the mammary gland assessed as either total area occupied by epithelium or as volume of epithelium. Moreover, the effects of corticosterone remained statistically significant even after adjustment of these values for differences among animals in body weight.

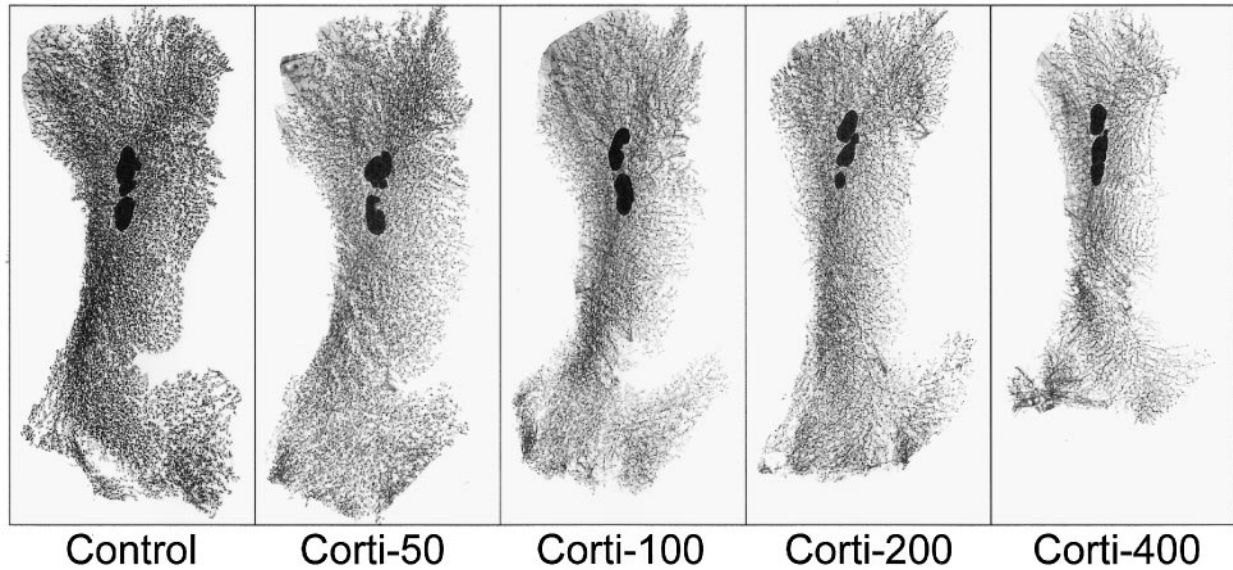


Fig. 2. Whole mounts of abdominal-inguinal mammary gland chains. Rats were fed either AIN-93G (control) diet or the AIN-93G supplemented with corticosterone at 50 (Corti-50), 100 (Corti-100), 200 (Corti-200) and 400 (Corti-400) mg corticosterone/kg diet. Whole mounts of the abdominal-inguinal mammary glands were prepared as described in Materials and methods. A representative whole mount from each experimental group is shown.

Table I. Effects of corticosterone administration on corticosterone levels in serum and urine, and adrenal gland weight of rat^a

	Control	Corti-50	Corti-100	Corti-200	Corti-400	P-value for trend
Urine-before ($\mu\text{g}/\text{kg}$ body wt/day)	3.19 ± 0.24^a	3.12 ± 0.11^a	3.11 ± 0.21^a	3.26 ± 0.14^a	3.20 ± 0.17^a	0.723
Urine-after ($\mu\text{g}/\text{kg}$ body wt/day)	2.56 ± 0.41^a	3.83 ± 0.48^a	4.78 ± 1.06^a	9.04 ± 0.54^b	18.40 ± 0.97^c	0.001
Serum-after (ng/ml)	188 ± 39^a	201 ± 41^a	223 ± 36^a	$379 \pm 85^{a,b}$	532 ± 88^b	0.001
Adrenal gland weight (mg)	30.4 ± 1.3^a	$26.5 \pm 1.0^{a,b}$	22.5 ± 1.3^b	13.6 ± 1.6^c	8.9 ± 0.8^d	0.001

^aEach value is the mean \pm SE. Data were analyzed by analysis of variance and regression analysis. *Post hoc* comparisons were made using Tukey's multiple-range test. Values with different superscript letters are significantly different ($P < 0.05$).

Table II. Effects of corticosterone administration on mammary optical density of rat^a

	Control	Corti-50	Corti-100	Corti-200	Corti-400	P-value for trend
Final body wt (g)	165 ± 6^a	165 ± 5^a	162 ± 4^a	156 ± 3^a	148 ± 5^a	0.003
Ductal extension (mm)	14.6 ± 0.4^a	$12.5 \pm 0.7^{a,b}$	$14.3 \pm 0.5^{a,b}$	$12.1 \pm 0.7^{a,b}$	11.8 ± 0.7^b	0.008
Ductal extension per 100 g body wt	8.9 ± 0.5^a	7.6 ± 0.4^a	8.8 ± 0.3^a	7.7 ± 0.4^a	8.0 ± 0.4^a	0.253
Total mammary gland area (cm^2)	10.4 ± 0.4^a	$9.8 \pm 0.4^{a,b}$	$9.3 \pm 0.3^{a,b}$	$9.3 \pm 0.2^{a,b}$	8.8 ± 0.4^b	0.001
Total mammary gland area/100 g body wt	6.3 ± 0.2^a	5.9 ± 0.2^a	5.8 ± 0.2^a	6.0 ± 0.2^a	5.9 ± 0.2^a	0.286
Epithelial area (cm^2)	3.6 ± 0.3^a	$3.0 \pm 0.3^{a,b}$	$2.9 \pm 0.2^{a,b}$	$2.9 \pm 0.1^{a,b}$	2.5 ± 0.2^b	0.001
Epithelial area per 100 g body wt	2.2 ± 0.2^a	$1.8 \pm 0.1^{a,b}$	$1.8 \pm 0.1^{a,b}$	$1.8 \pm 0.1^{a,b}$	1.7 ± 0.1^b	0.007
Epithelial density	1.31 ± 0.16^a	$1.19 \pm 0.14^{a,b}$	$1.06 \pm 0.03^{a,b,c}$	$0.91 \pm 0.03^{b,c}$	0.77 ± 0.05^c	0.001
Epithelial density/100 g body wt	0.79 ± 0.09^a	$0.71 \pm 0.07^{a,b}$	$0.65 \pm 0.01^{a,b,c}$	$0.58 \pm 0.02^{b,c}$	0.52 ± 0.02^c	0.001

^aEach value is mean \pm SE. Data were analyzed by analysis of variance and regression analysis. *Post hoc* comparisons were made using Tukey's multiple-range test. Values with different superscript letters are significantly different ($P < 0.05$).

p27 expression in mammary ductal epithelium

Figure 3 shows the effect of corticosterone treatment on the number of mammary ductal epithelial cells staining positive for p27/kip1 (Figure 3A) and on the estimated amount of p27 protein per positive staining cell (Figure 3B) in mammary ducts. Corticosterone treatment resulted in a dose-dependent increase in the proportion of cells expressing p27 ($P < 0.0001$). The magnitude of increased expression ranged from ~2- to 14-fold. The same pattern of effect as observed for the proportion of positive staining cells was noted for the estimated amount of p27 protein per positive staining cell.

Overall correlations

Figure 4 summarizes a series of correlation analyses among the amount of corticosterone in the diet, the levels of cortical steroids in urine and serum, the number of mammary epithelial cells staining positive for p27, the estimated amount of p27 protein per positive staining cell, and the area and density of mammary gland epithelium. Correlations were positive and statistically significant ($P < 0.05$) for dietary corticosterone and cortical steroid concentration in serum or urine and between each of these variables and p27 expression (both the number of cells expressing p27 protein and the amount of p27

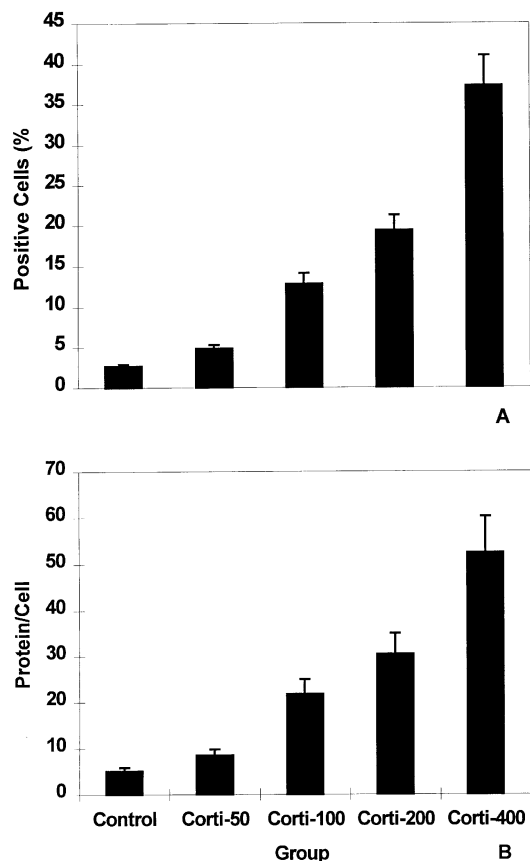


Fig. 3. p27 protein expression in mammary duct epithelium of rats treated with corticosterone in diet. Values are means with SE bar. (A) The percentage of cells staining positive for p27; (B) the amount of p27 in positive-stained cells. A significant increase in p27 protein was found with elevated corticosterone in diet (Kruskal–Wallis rank test, $P < 0.0001$).

protein per cell). Negative correlations that were statistically significant ($P < 0.05$) were observed between dietary corticosterone or cortical steroids in serum or urine and the area occupied by or density of mammary gland epithelium.

Discussion

The restriction of energy to an animal inhibits carcinogenesis in various organ sites including the mammary gland, and is associated with other beneficial effects (1,19,20). For this reason, important insights may be gained by understanding the mechanism(s) by which energy restriction exerts these effects. Our laboratory has recently reported evidence indicating that energy restriction may inhibit carcinogenesis by arresting cell cycle progression (21). Moreover, the evidence presented (1,11,21) indicates that corticosterones may either be a marker of this effect or be directly involved in arresting cell cycle progression. Because energy restriction has been shown to alter the levels of many hormones (2) we undertook the experiment reported in this paper to develop a model for systematically evaluating the effects of cortical steroids on mammary carcinogenesis. Several approaches to cortical steroid administration were considered. These included the use of time release pellets or osmotic pumps, stomach intubation and injection. However, the dietary approach was selected in view of the nibbling type feeding pattern of rats, the lack of invasiveness of the approach, the self-regulated adjustment of dietary intake to energy requirements and the ease of

supplementation. The data in Table I show the effectiveness of the dietary approach in increasing both serum and urinary levels of corticosterone detected by radioimmunoassay. If these data are compared with data presented in Table III, which summarizes in tabular form the information reported previously (1), it is clear that feeding 50, 100 and 200 p.p.m. corticosterone in the diet resulted in urinary levels of cortical steroids comparable with those observed in female rats fed 90, 80 or 60% of the energy consumed by rats fed *ad libitum* (1). Thus, these data provide the basis for the design of experiments to further investigate the effects of specific cortical steroids on mammary carcinogenesis. It is also important to comment on the choice of the adrenal cortical steroid investigated. The primary glucocorticoid is corticosterone in rats and cortisol in humans (22). Thus in these initial studies, we have chosen to focus on the effects of a cortical steroid metabolite that is physiologically relevant in the rat. Nevertheless, it is of interest that our laboratory has recently determined that dehydroepiandrosterone, a major adrenal cortical steroid in humans but not in rats, and at similar dietary concentrations to the dietary concentrations of corticosterone used in this study, was effective in inhibiting mammary carcinogenesis (H.J.Thompson, unpublished results). This observation provides further support for the use of the described dietary approach to investigate the role of specific cortical steroid metabolites in inhibiting mammary carcinogenesis.

The approach that was used to estimate the effect of corticosterone on mammary gland development has been recently reported by our laboratory (23,24). It is notable that this approach parallels current efforts to quantify breast density in women as a surrogate marker for breast cancer risk (25). What is predicted in women is that there is a direct relationship between breast density and cancer risk (25,26). Thus the observation that breast density measured either as area occupied by mammary epithelium or as the estimated volume of mammary epithelium was reduced by dietary corticosterone administration and that this effect was independent of differences in body weight among animals, and is both parallel to the effects of energy restriction on mammary gland density and is consistent with our hypothesis that cortical steroids account, at least in part, for the cancer inhibitory effect of energy restriction (23). Nevertheless, a comparison of the data on mammary development presented in Table II with corresponding data reported elsewhere (23) and summarized in Table III shows that energy restriction, at its highest level, resulted in a 56% reduction in mammary epithelial area, whereas the highest level of corticosterone reduced epithelial area by only 31%. Thus as we recently suggested in Refs (1,21,23), it is likely that energy restriction acts concomitantly on metabolic pathways regulated not only by adrenal cortical steroids, but also by insulin and insulin-related growth factors. Thus, the effects of energy restriction are unlikely to be mediated via cortical steroids alone. Whether or not dietary administration of corticosterone modulated the secretion and/or metabolism of insulin and related growth factors remains to be determined.

The data shown in Table II indicate that corticosterone regulated mammary gland size homeostasis in a manner parallel to the effects of energy restriction on mammary gland development. This prompted us to determine if the amount of p27 protein in mammary ductal epithelial cells was increased by dietary corticosterone. As shown in Figure 4 there was a strong dose-dependent increase in the number of cells staining

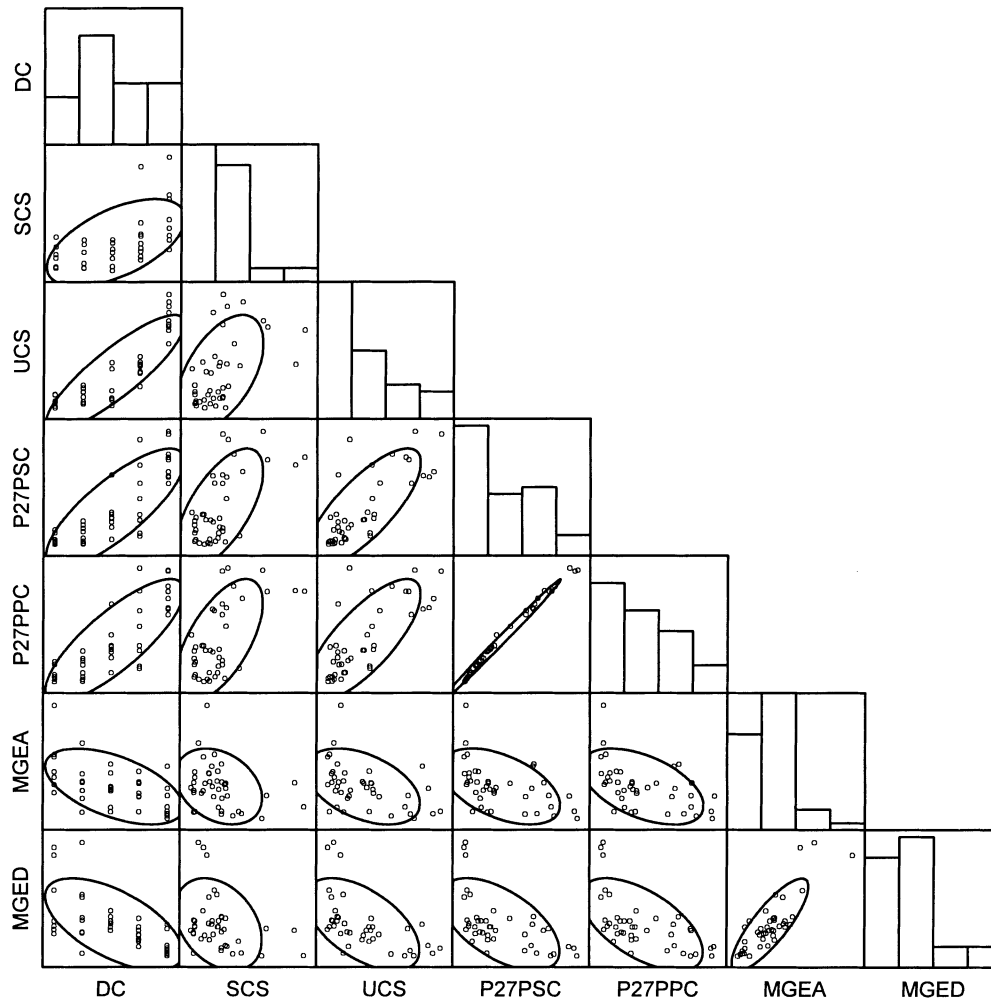


Fig. 4. Correlation analyses among dietary corticosterone (DC), serum cortical steroid (SCS), urinary cortical steroid (UCS), p27 positively stained cells (P27PSC), p27 protein per cell (P27PPC), mammary gland epithelial area (MGEA) and mammary gland epithelial density (MGED). All of the correlations among them were statistically significant ($P < 0.05$ to $P < 0.001$). The ellipses are the 75% bivariate distribution of Gaussian (normal). The shape of the ellipse is an indication of the independence of the data plotted. The more independent the plotted data are of one another, the more circular the ellipse; whereas a narrow ellipse indicates a high degree of dependence (relatedness) of the data plotted.

Table III. Effects of energy restriction on urinary cortical steroid excretion and mammary epithelial area of rat^a

	Control	90% RF ^b	80% RF	60% RF
Urine-after ($\mu\text{g}/\text{kg}$ body wt/day)	3.20 ± 0.27^a	$5.04 \pm 0.53^{a,b}$	6.97 ± 0.52^b	11.59 ± 1.64^c
Epithelial area (cm^2)	3.93 ± 0.10^a	2.92 ± 0.08^b	2.49 ± 0.10^c	1.72 ± 0.07^d

^aEach value is mean \pm SE. Data were analyzed by analysis of variance and regression analysis. *Post hoc* comparisons were made using Tukey's multiple-range test. Values with different superscript letters are significantly different ($P < 0.05$).

^bAll rats were meal fed. Animals in the control group were fed *ad libitum* at each meal; calorie restricted-fed rats (RF) were fed 90, 80 or 60% of the *ad libitum* intake.

positive for p27 and for the estimated amount of p27 protein per positive-staining cell with increasing level of corticosterone. Based on this result we next examined the relationship among animals, irrespective of their dietary treatment, between p27-positive staining and estimated amount of mammary epithelium. We predicted that if increased p27 was indicative of growth arrest, then the amount of mammary epithelium estimated by both mammary gland epithelial area and density would be reduced as the percentage of ductal mammary epithelial cells staining positive for p27 increased. We obtained strong statistical support for this hypothesis as shown graphic-

ally in Figure 4. The Spearman correlation coefficient was $r = -0.66$, $P < 0.01$. These findings further support the hypothesis that corticosterone, as well as energy restriction, negatively regulate the growth of mammary gland by inducing cell cycle arrest via affecting cellular levels of p27.

In summary, our current working hypothesis is that energy restriction acts to inhibit carcinogenesis via a mechanism involving adrenal cortical steroids and that a target of this effect is the control of cell cycle progression. The family of p21 proteins, of which p27 is a member, represents a potential target of the effects of energy restriction.

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