

Energy availability and mammary carcinogenesis: effects of calorie restriction and exercise

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The purpose of this experiment was to compare the carcinogenic response in the mammary gland among groups of rats whose energy metabolism had been modulated by restricting dietary calories and/or by increasing energy expenditure via exercise. Female F344 rats ($n = 132$) were injected i.p. with 1-methyl-1-nitrosomethylurea (50 mg/kg at 50 and 57 days of age) and were randomized into one of four treatment groups: (i) unrestricted, sedentary; (ii) calorie-restricted, sedentary; (iii) unrestricted, exercised; (iv) calorie-restricted, exercised. The targeted level of calorie-restricted was 20% and exercise was achieved by treadmill-running (20 m/min at a 15% grade for 30 min, 5 days/week). During the 20.5 week study, rats were palpated twice a week for detection of mammary tumors and urine was collected for determination of 24-h cortical steroid excretion. At the end of the study, all mammary lesions were histologically classified. Carcass composition and carcass energy were determined. Mammary carcinogenesis was inhibited among calorie-restricted, sedentary rats compared with unrestricted, sedentary rats (79% inhibition, $P < 0.001$). No inhibition of carcinogenesis was observed among exercised rats (unrestricted or calorie-restricted) relative to the unrestricted, sedentary rats. Within the present experimental design, exercise had no effect on carcinogenesis despite significant reductions of carcass fat and carcass energy among both groups of rats that exercised. Cortical steroid level was significantly higher only in calorie-restricted, sedentary rats ($P < 0.05$). These results do not support the hypothesis that reductions of body weight gain, carcass fat or carcass energy are sufficient conditions to inhibit mammary carcinogenesis. The results do suggest that changes in urinary cortical steroid excretion may predict whether an energy-related intervention is likely to alter mammary carcinogenesis.

Introduction

Using widely accepted animal models for breast cancer, it has been consistently observed that the occurrence of spontaneous, chemically- and virally-induced mammary carcinogenesis can be inhibited by restricting the intake of dietary calories (1–5). The specific changes induced by calorie restriction, which are responsible for the inhibition of mammary carcinogenesis, are unknown. As yet, it is unclear whether the most apparent

*Abbreviations: IACUC, Animal Care and Use Committee; MNU, 1-methyl-1-nitrosourea.

effects of calorie restriction, namely: reduced food intake, retarded rate of body weight gain, decreased carcass fat accumulation or decreased carcass energy accumulation account for the cancer inhibitory activity of caloric restriction.

Besides reducing energy intake via calorie restriction, the amount of energy available to an organism to support body weight gain, carcass fat accumulation and carcass energy accumulation can be reduced by increasing energy expenditure. Increased physical exercise is one of the principal means to increase energy expenditure. Previous research on the effects of exercise on mammary carcinogenesis, unlike past research on the effects of calorie restriction, have produced variable results (6). Exercise has been reported to both inhibit and to enhance mammary carcinogenesis (7–11). The reasons for the range of effects observed are unclear, although differences in exercise type, intensity, and duration are likely to be involved (11,12). It is also important to note that, while exercise is generally observed to increase energy expenditure and reduce body weight gain, carcass fat and carcass energy accumulation, these effects are variable, particularly in female animals (8–11). It has been observed in some studies that female rats exposed to exercise training sometimes respond by increasing food consumption and increasing body weight (13–15). It is unclear whether the effect of exercise on carcinogenesis varies depending on its effect on energy status. Few studies examining the effect of exercise on mammary carcinogenesis have also measured the effect of exercise on food intake, body weight, carcass composition and carcass energy.

The purpose of this study was to compare the carcinogenic response in the mammary gland among groups of female rats in which energy availability was reduced by: (i) calorie restriction; (ii) increased exercise; or (iii) a combination of exercise and calorie restriction. Energy status, during the experimental period, was estimated by measuring daily food intake and body weight and, at the end of the experiment, by assessing carcass composition and carcass energy. It was hypothesized that limiting the energy available, by calorie restriction or by exercise, would inhibit mammary carcinogenesis and that a comparable reduction of available energy by the combination of exercise and calorie restriction, would be at least as effective at inhibiting carcinogenesis as calorie restriction or exercise alone.

Materials and methods

Animals

Female F-344 rats ($n = 132$) were obtained from Taconic Farms (Germantown, NY) at 21 days of age and were housed individually in stainless steel cages with wire-mesh bottoms. Rooms were maintained at $22 \pm 1^\circ\text{C}$ with 50% relative humidity and a 12-h light/12-h dark cycle. The work reported was reviewed and approved by AMC's Institutional Animal Care and Use Committee (IACUC*) and conducted according to IACUC guidelines.

Experimental design

At 50 and 57 days of age, rats were injected i.p. with 50 mg 1-methyl-1-nitrosourea (MNU)/kg body weight as previously described (11,16). MNU was purchased from Ash Stevens, Detroit, MI. The two-injection protocol

was chosen to increase the sensitivity of the carcinogenesis bioassay. This decision was based on recently published exercise and mammary cancer data that demonstrated the need for this approach in order to distinguish between exercise- and calorie-related treatment effects (10,11). In order to confine treatment effects to the post-initiation stage of carcinogenesis, rats did not begin exercise and/or calorie restriction until 7 days after the second dose of carcinogen was administered. At 64 days of age, rats were stratified by weight and randomly assigned to one of four groups: (i) unrestricted, sedentary ($n = 30$); (ii) calorie-restricted, sedentary ($n = 30$); (iii) unrestricted, exercised ($n = 33$); and (iv) calorie-restricted, exercised ($n = 34$). As discussed below, meal frequency and meal timing was consistent for all rats. The unrestricted, sedentary rat group was operationally defined as the control group. One rat was not compliant with the exercise protocol and two more rats were injured during exercise; these animals were excluded from the study and its analysis. The duration of the experiment was 20.5 weeks.

Diet composition and meal-feeding

From days 21 to 28, all rats were fed a purified diet *ad libitum*. The composition of the diet (AIN-76A) is shown in the first column in Table I (9,17,18). On day 28, meal-feeding was begun for all rats. The meal-feeding protocol allowed all rats to consume an unlimited quantity of the AIN-76A diet, but access to the diet was limited to two, 3-h meal periods: 06:00–09:00 h and 14:00–17:00 h. This feeding protocol was used to ensure that the conditions of meal frequency and hours of fasting would be similar between unrestricted and calorie-restricted treatment groups. Calorie restriction began in the two restricted groups when rats were 64 days of age. The nutrient per kcal ratio of the diet fed to restricted rat groups was increased (see Table I). This level of increase kept the differences in the intakes of fat, protein, fiber, vitamins and minerals among groups of rats, to <2% per day. Calorie-restricted, sedentary rats were provided with 80% of the previous day's average intake of that of the unrestricted, sedentary rats. In an effort to provide the calorie-restricted, exercise rats and the calorie restricted, sedentary rats with a similar relative energy restriction, the calorie-restricted exercise rats were given 1 g more of the diet (3.913 kcal) than calorie-restricted, sedentary rats on exercise days (5 days/week) and 0.2 g more of the diet (0.783 kcal) on non-exercise days (2 days/ week). These amounts were derived from a pilot study (data not shown).

Exercise training

In order to ensure that the physical environment of sedentary and exercise-trained animals was similar, all rats were housed in the rooms with the treadmills. Additionally, sedentary rats were 'sham' trained on stationary treadmills. Sedentary rats were placed on a stationary treadmill for 30 min for 5 days/week at 0 m/min on a 15% grade, during the same time that exercise rats were running. Exercised rats were progressively trained to run 20 m/min at a 15% grade for 30 min for 5 days/week. Exercise training began at 0 m/min, 0% grade, for 5 min and was gradually increased in intensity and in duration for 7 training days until the 20 m/min, 15% grade, for 30 min level was reached. Exercise was conducted during lights-on hours.

Measurement of corticosterone

During the experiment, findings from a parallel but independent study suggested that changes in immunoreactive urinary cortical steroids correlated with the cancer-inhibitory effects of calorie restriction (19). Based on these observations, we compared the levels of cortical steroid among groups in the present study. Two weeks before the completion of the study, a sample of randomly-selected rats ($n = 18$ rats per treatment group) were placed in metabolic cages and urine was collected for 24 h. The timing of the collection was such that the exercise rats had trained the day of collection and had trained the previous 3–4 consecutive days. Exercise rats were only removed from the metabolic cages during the 30 min needed to complete the exercise protocol. Urine was assayed for immunoreactive cortical steroids via a direct radioimmunoassay specific for corticosterone (ICN Biomedicals, Inc., Costa Mesa, CA).

Necropsy

All rats were euthanized by CO₂ inhalation at 20.5 weeks post-carcinogen. Rats were skinned and the skin was examined under translucent light. All grossly detectable mammary gland lesions were excised and imbedded in paraffin for processing for histopathological classification (20).

Measurement of carcass composition

Nine tumor-free rats per group were selected for determination of carcass composition. The selection was made such that the difference between the average body weight of a group and the average body weight of the sampled rats was <2 g. In addition, at the beginning of the study, five rats with an average body weight equal to the mean weight of the rats just prior to randomization (63 days), were killed for the estimation of initial carcass energy. After removing intestinal contents, the carcasses were weighed

Table I. Diet formulations

Ingredients ^a	Diet compositions (%)	
	Unrestricted	Calorie restricted ^b
Corn oil	5.000	5.882
Casein	20.000	23.530
DL-Methionine	0.300	0.353
Cerelose ^c	32.500	29.411
Corn starch ^c	32.500	29.412
Fiber	5.000	5.882
AIN-76 mineral mix ^d	0.500	4.118
AIN-76 vitamin mix ^d	1.000	1.177
Choline bitartrate ^d	0.200	0.235
Total	100.0	100.0
kcal density/g	3.9260	3.913
% kcal from fat	11.462	13.529

^aIngredients of the grade specified in (17).

^bThe density of nutrients (except carbohydrate) was enhanced 15% so that despite reduced intake, the calorie restriction rats consumed approximately the same quantity of nutrients as unrestricted rats.

^cSource of carbohydrate was modified as suggested in (18) to avoid complications that can result from feeding a high sucrose diet in long-term experiments.

^dAIN-76A formulations specified in (17) as modified in (18).

and homogenized. Warren Laboratories (Greeley, CO) determined carcass percentages of moisture (lyophilized weight divided by fresh weight), protein (estimated by the Kjeldahl method), crude fat (the ether extractable residue) and ash (the residue left after ignition in a muffled furnace). Cumulative carcass energy gain was calculated by applying the values of 5.319 kcal/g of carcass protein and 8.895 kcal/g of carcass fat (21) for each of the sampled rats and subtracting the mean carcass energy value (221 ± 15 kcal) obtained for the five rats killed just before beginning treatments.

Statistical analysis

Initially, a two-by-two factorial design was used to compare the effects of two levels of exercise (sedentary and running) and two levels of energy status (unrestricted and calorie-restricted). As shown in the Results section, a significant interaction was observed ($P < 0.05$) suggesting that the effect of calorie restriction on mammary carcinogenesis was different for sedentary rats compared with running rats. Subsequent statistical procedures made simple comparisons among the four rat groups treating the experiment as a one-way design with four levels. Carcass data and cortical steroid hormone level were analyzed using one-way analysis of variance. The method of testing the contrasts among levels incorporated Bonferroni's inequality (22). Differences among groups in the final incidence of cancer were evaluated by chi-square analysis (23). Mantel's life table procedure (log-rank test) was used to determine differences in cancer latency (time before the appearance of detectable mammary cancer) (24). Poisson regression analyses were used to detect significant treatment effects and cortical steroid effects on cancer multiplicity (22). These analyses, similar to multiple regression analysis, allowed determination of the relative contribution of treatment group and cortical steroid excretion as explanatory variables for mammary carcinogenesis. The ability to analyze the data in this way was useful because a finding that both treatment group and cortical hormone level explain a significant portion of the variance in mammary cancer supports the possibility that treatment effects may be mediated by cortical steroid hormones.

Results

Daily measures of energy-related variables

Table II and Figure 1 show the effects of the various treatments on energy metabolism-related variables. Unrestricted, sedentary rats consumed, on average, 1070 g of diet during the 20.5 week experiment. The intent was to restrict calorie-restricted, sedentary rats to 80% of the amount eaten by the unrestricted, sedentary group, i.e. a 20% level of restriction. The measured level of the calorie restriction was 18.6%. The intent was also, taking into account exercise energy expenditure, to match the energy restriction of calorie-restricted, exercised rats to that

Table II. Effects of calorie restriction and/or exercise on energy-related variables

Treatment		Cumulative weight gain		Cumulative diet intake		Diet efficiency weight gain/diet intake	
Calorie	Exercise	(g)	% control	(g)	% control	(g/g)	% control
Unrestricted	Sedentary	57 ± 0.33 ^a	100	1070 ± 3.3 ^a	100	0.053 ± 0.0002 ^a	100
Restricted	Sedentary	32 ± 0.23 ^b	60	872 ± 0.73 ^b	82	0.037 ± 0.0003 ^b	67
Unrestricted	Exercise	55 ± 0.31 ^a	97	1137 ± 3.7 ^a	106	0.048 ± 0.0003 ^a	90
Restricted	Exercise	36 ± 0.31 ^b	63	964 ± 0.88 ^b	90	0.037 ± 0.0003 ^b	67

Each value is the mean ± SEM for 18.5 weeks including all rats living until study completion.

^{a,b}Values with in a column with different superscripts are significantly different ($P < 0.05$).

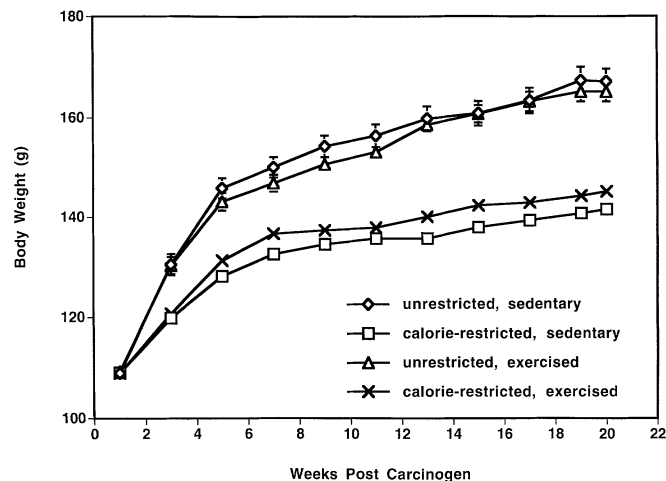


Fig. 1. Effects of calorie-restriction and/or exercise on the rate of body weight gain. Values are means ± SEM. Note: For calorie-restricted, sedentary and for calorie-restricted, exercise rats the standard error bars are smaller than the symbols used to indicate treatment group.

of the calorie-restricted, sedentary rats. In a preliminary study (data not shown), it was observed that, in order to achieve a similar relative energy restriction in calorie-restricted, exercised animals compared with calorie-restricted, sedentary rats, the exercise group should receive more food (to compensate for exercise energy expenditure) and weigh slightly more (exercise produced increased carcass protein and decreased carcass fat). The feeding regimen described in the present study was based on the pilot study findings. The actual level of restriction for the calorie-restricted, exercise rats was determined by adjusting the total amount of diet consumed, by the calorie-restricted, exercise group by the difference in food intake of the unrestricted, sedentary and unrestricted, exercise rats. The adjusted amount was divided by the amount consumed by the sedentary unrestricted rats. Using this method of calculation the estimated level of restriction was 16.5%, a level that was not significantly different from the level of restriction to which sedentary rats were subjected.

The cumulative feed efficiency ratios of both groups of calorie-restricted rats were similar, but statistically lower than both groups of unrestricted rats ($P < 0.05$). The pattern of body weight gain for each group is shown in Figure 1. The growth rates of both groups of rats that were calorie-restricted were similar, but significantly lower than those of unrestricted rats ($P < 0.01$).

Carcass energy variables

Despite the apparent similarities between the calorie-restricted groups compared with the unrestricted groups suggested by

the data in Table II and Figure 1, the carcass composition data presented in Tables III and IV revealed a different compositional pattern. Percent carcass fat was similar between sedentary groups of rats (unrestricted versus calorie-restricted) and was also similar between exercised rats (unrestricted versus calorie-restricted). However, percent carcass fat was significantly reduced by exercise. Within the categories of sedentary or exercised rats, the calorie restriction did not alter relative body composition but did significantly reduce absolute levels of carcass fat, carcass protein and carcass energy. The proportion of dietary calories retained as carcass calories, was determined using data shown in Table IV; it was calculated as the ratio of total increase in carcass energy divided by total dietary energy intake during the 20.5 weeks. The proportion of dietary calories retained as carcass calories was dramatically reduced by exercise energy expenditure. Calorie restriction, for the sedentary or exercised group was also associated with a reduction in this ratio, but the magnitude of the reduction was smaller.

Mammary cancer

The administration of MNU resulted in the induction of malignant and benign mammary tumors. However, >99% of the tumors were carcinomas. The data reported are based on only those tumors classified histologically as mammary gland adenocarcinomas. The effects of calorie restriction, exercise, and the combination of these factors on incidence and multiplicity of mammary carcinomas are shown in Table V and Figure 2. Calorie-restriction alone, but not exercise or exercise plus calorie-restriction, reduced the incidence ($P < 0.01$) and multiplicity ($P < 0.01$) of mammary carcinoma and prolonged the latency period ($P < 0.05$) relative to the control group.

Urinary cortical steroid

The effect of treatment on 24 h urinary excretion of immunoreactive cortical steroid hormone is shown in Table IV and Figure 3. Cortical steroid excretion was two-fold higher in calorie-restricted, sedentary rats. There were no significant differences in cortical steroid excretion among the exercised rats (unrestricted or calorie-restricted) and control. Results of the Poisson regression of tumor multiplicity showed that among the 72 rats in which the hormone was measured, a significantly greater proportion of the variance in mammary cancer could be explained when both hormone level and treatment group were considered in the regression, compared with the regression in which only the treatment group was used as an explanatory variable.

Discussion

The purpose of the present investigation was to test the hypothesis that limiting the energy available to an animal by

Table III. Effects of calorie restriction and/or exercise on carcass composition

Treatment		Compositions (%)			
Calorie	Exercise	Fat	Moisture	Protein	Ash
Unrestricted	Sedentary	19.4 ± 0.36 ^a	57.6 ± 0.22 ^a	19.5 ± 0.16 ^a	3.9 ± 0.04 ^a
Restricted	Sedentary	18.7 ± 0.29 ^a	57.9 ± 0.20 ^a	20.2 ± 0.11 ^a	3.9 ± 0.04 ^a
Unrestricted	Exercise	11.6 ± 0.14 ^b	62.7 ± 0.11 ^b	21.6 ± 0.09 ^b	4.3 ± 0.04 ^b
Restricted	Exercise	11.7 ± 0.20 ^b	63.2 ± 0.17 ^b	21.7 ± 0.04 ^b	4.1 ± 0.04 ^{a,b}

^{a,b}Values within a column with different letter superscripts are significantly different ($P < 0.05$). Each value is the mean ± SEM of nine rats.

Table IV. Effects of calorie restriction and/or exercise on carcass energy gain and cortical steroid levels

Treatment		Carcass energy gain ^a		Carcass gain ^a /Diet intake		Cortical steroid ^b	
Calorie	Exercise	(kcal)	% control	(kcal/kcal)	% control	µg/kg per 24 h	% control
Unrestricted	Sedentary	247 ± 6.9 ^c	100	0.059	100	1.33 ± 0.15 ^c	100
Restricted	Sedentary	172 ± 3.7 ^d	69	0.050	85	3.78 ± 0.65 ^d	284
Unrestricted	Exercise	138 ± 2.7 ^{c,d}	56	0.031	53	1.51 ± 0.11 ^c	114
Restricted	Exercise	98 ± 2.1 ^e	40	0.026	44	1.92 ± 0.24 ^c	144

^aEach value is the mean ± SEM of nine rats.

^bEach value is the mean ± SEM of 18 rats.

^{c,d,e}Values within a column with different letter superscripts are significantly different ($P < 0.05$).

Table V. Effects of calorie restriction and exercise on mammary cancer

Treatment		<i>n</i>	Cancer incidence		Cancer multiplicity	
Calorie	Exercise		%	% control	cancers/rat	% control
Unrestricted	Sedentary	30	23.3 ^a	100	0.367 ^a	100
Restricted	Sedentary	30	6.7 ^b	29	0.067 ^b	29
Unrestricted	Exercise	32	28.1 ^a	121	0.469 ^a	139
Restricted	Exercise	32	34.4 ^a	148	0.500 ^a	148

^{a,b}Values within a column with different letter superscripts are significantly different ($P < 0.05$).

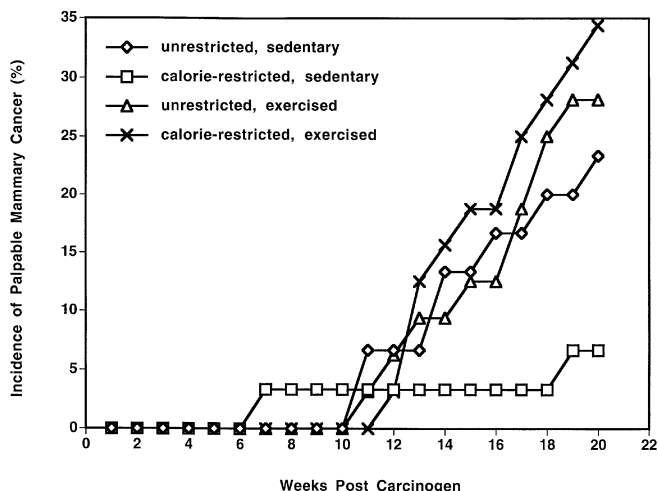


Fig. 2. Effects of calorie-restriction and/or exercise on the incidence of palpable mammary carcinomas as a function of time after carcinogen administration.

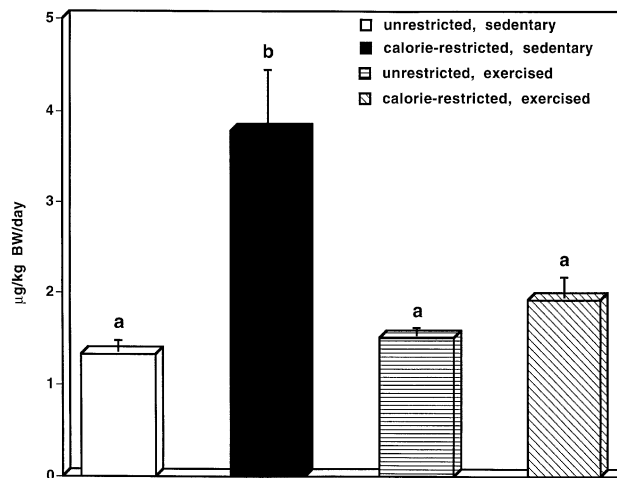


Fig. 3. Effects of calorie-restriction and/or exercise on urinary excretion of cortical steroids. Values are means ± SEM.

calorie restriction or by exercise would inhibit mammary carcinogenesis, and that a comparable reduction of available energy by the combination of exercise and calorie restriction, would be at least as effective at inhibiting carcinogenesis as calorie restriction or exercise alone. In order to evaluate this hypothesis it was deemed essential to use an experimental design that differed from those used in the majority of previous studies involving calorie restriction or exercise and mammary carcinogenesis in that the pattern of food intake was controlled by meal-feeding all animals. It is well established that the pattern in which a given number of calories are consumed influences the metabolic handling of nutrients (25). In turn, the metabolic handling of nutrients may influence other factors important to the process of carcinogenesis (26,27). The use of a meal-feeding protocol eliminated differences among groups in food intake patterns. Without meal-feeding, unrestricted rats consume diet in numerous, frequent, meals and avoid prolonged fasting while calorie-restricted rats completely consume their diet soon after it is presented and fast between feedings. With meal-feeding, these differences in fasting and re-feeding were eliminated and the effects of dietary calories could be distinguished from the effects of food-intake patterns. Within this design, the hypothesis that limiting available energy to the animal by either decreasing energy intake and/or increasing energy expenditure would inhibit mammary carcinogenesis, was not supported (Figure 2 and Table V). Whereas calorie restriction inhibited mammary carcinogenesis in sedentary rats, inhibition of mammary carcinogenesis was not observed either in unrestricted, exercised or in calorie-restricted, exercised rats compared with unrestricted, sedentary (control) rats.

Close inspection of the energy-related data shown in Tables II, III and IV reveals several differences in the effects achieved by exercise compared with the effects achieved by calorie restriction. The greater weight gain, food intake and feed efficiency of unrestricted, exercise rats versus calorie-restricted, sedentary rats might be considered to be consistent with the comparatively greater carcinogenic response observed in the exercised rats (30). However, the lower carcass fat and reduced carcass energy observed in unrestricted, exercise compared with unrestricted, sedentary rats has been associated with protection against carcinogenesis (31). This conundrum was accentuated by inspection of the data from the calorie-restricted, exercised rats. These animals had body weight gains comparable to sedentary calorie-restricted rats but lower levels of body fat and carcass energy. Nevertheless, there was no inhibition of the carcinogenic response in these exercised rats, relative to the unrestricted, sedentary rats. These data imply that increasing energy expenditure via exercise may actually interfere with the inhibition of carcinogenesis associated with calorie restriction. While we are unaware of a precedent for this observation in the exercise-carcinogenesis literature, these results are consistent with findings from studies of longevity and aging. Similar to our findings, investigators in this area have reported that the combination of exercise and calorie restriction is less beneficial than calorie restriction alone (28–30).

A potential explanation for the different effects of caloric restriction versus exercise on the carcinogenic response may relate to the differences in urinary cortical steroid excretion among groups (Table IV and Figure 3). In several reports, the inhibition of carcinogenesis by energy restriction has been shown to be associated with increased adrenal cortical activity (31–33). In this study, we tested the hypothesis that a change

in adrenal function, more specifically increased excretion of cortical steroids, would be associated with the cancer-inhibitory activity of energy restriction. Due to the time-averaged, non-invasive nature of urine collection, measurement of cortical steroid levels in 24 h urine collections was used to assess adrenocortical activity. Urinary cortical steroid measurement is a well-established method of estimating adrenal cortical activity that largely avoids confounding issues associated with serum cortisol measurement, specifically, episodic secretion, diurnal variation and sample collection induced changes (34–36). In this experiment a significantly higher level of urinary cortisol was observed in the group of animals with the lowest cancer incidence (calorie-restricted, sedentary). Additionally, variations among animals in urinary cortisol accounted for a significant portion of the variation in the mean number of cancers per rat and an animal's level of urinary cortisol was predictive of whether it was tumor-bearing or tumor-free. The lack of significantly increased cortisol among the exercised groups compared with the control was unanticipated since exercise has been reported to stimulate adrenal cortical activity (37). It is possible that the meal-feeding protocol affected the animal's adrenal cortical response to exercise such that the intensity and/or duration of exercise used in this experiment was not sufficient to significantly increase total adrenal cortisol production.

In conclusion, these results indicate that reductions in body weight gain, carcass fat or carcass energy are not sufficient to inhibit mammary carcinogenesis and accentuate the need for further investigation of the interactions between the processes used to maintain energy homeostasis and the processes that may inhibit mammary carcinogenesis. Whether adrenal cortical steroids and/or other metabolic hormones are involved is currently under investigation. At this point, it appears that urinary cortisol levels may serve as a useful marker indicating whether an energy-related intervention is likely to produce cancer inhibition; this marker could serve to guide mechanistic inquiries of this complex phenomenon.

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